Directed differentiation of mouse embryonic stem cells into neocortical output neurons

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Abstract

During development of the neocortex, many diverse projection neuron subtypes are generated under regulation of cell-extrinsic and cell-intrinsic controls. One broad projection neuron class, corticofugal projection neurons (CFuPN), is the primary output neuron population of the neocortex. CFuPN axons innervate sub-cortical targets including thalamus, striatum, brainstem, and spinal cord.

The broad class of CFuPN includes multiple subtypes of projection neurons, residing and intermixed in distinct neocortical layers and areas, with distinct axonal targets. Corticospinal motor neurons (CSMN) are one clinically important corticofugal subtype; they reside in motor cortex layer V, and project axons to the spinal cord to control motor function. CSMN degenerate in amyotrophic lateral sclerosis (ALS), along with spinal motor neurons, and CSMN axonal damage after spinal cord injury (SCI) is central to ensuing loss of motor function. No effective therapies exist for ALS or SCI; the inability to produce CSMN (and CFuPN) *in vitro* is a significant roadblock to developing new treatments.

Embryonic stem (ES) cells have theoretical potential to generate CFuPN *in vitro*. However, existing ES cell protocols are not directed toward generating specific classes, types, or subtypes of neocortical projection neurons.

Work presented in this dissertation makes progress toward characterization and directed differentiation of CFuPN derived from mouse ES cells. First, I show that existing protocols produce heterogeneous, neocortical-like neurons that are immature and “stalled” at a stage resembling mid-embryonic differentiation *in vivo*. These analyses apply multiple criteria

derived from an emerging understanding of CFuPN developmental biology. Second, I build on that foundation to promote CFuPN-specific differentiation among these “stalled” neocortical-like neurons, using recently-developed synthetic modified mRNA and high-content screening technologies. Finally, I enrich for ES-derived CFuPN by sequentially and transiently expressing critical forebrain, neocortex, and CFuPN-specific transcription factors; when micro-transplanted into neonatal neocortex, these neurons appropriately innervate specific targets in the thalamus and midbrain.

Together, biological and technical approaches presented in this dissertation both rigorously characterize ES-derived neuronal identity, and identify novel strategies to promote CFuPN differentiation. This work will enable further advances in the broader field of *in vitro* neurodegenerative disease modeling, particularly toward developing new treatments for ALS and SCI.

Table of Contents

Figures and Table

Abbreviations

I-VI	neocortical layers I-VI
CFuPN	corticofugal projection neurons
CPN	callosal projection neurons
CSMN	corticospinal motor neurons
CTPN	corticotectal projection neurons
CThPN	corticothalamic projection neurons
E	embryonic day
ES	embryonic stem
IC	internal capsule
iN	induced neuronal
iPS	induced pluripotent stem
modRNA	synthetic modified mRNA
NPC	neural progenitor cell
P	postnatal day
SCPN	subcerebral projection neurons
Str	striatum
Th	thalamus
VZ	ventricular zone

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To patients who will benefit from this work

Chapter 1

Introduction

1.1 Overview

The adult mammalian neocortex has the extraordinary capacity for higher-order cognitive functions and for orchestrating intricate motor behaviors, while continuously integrating feedback from major modalities of sensory input. In humans, the neocortex alone comprises over two-thirds the neuronal mass of the entire nervous system, and three-quarters of all synaptic connections (Rakic, 1988). Given the breadth of neocortical function and connectivity within the nervous system, it is not surprising that the neocortex is composed of hundreds of highly specialized neuronal subtypes with distinct connectivity, gene expression, and physiology (Molyneaux *et al.*, 2007; Gord and Bernardo, 2011).

The incredible neuronal diversity contained within the adult neocortex progressively emerges during an extended course of development (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). Neocortical projection neurons are among the very first neurons to populate the embryonic neocortex and are classified by their distinct axonal projections to multiple targets within or outside the neocortex (**Figure 1.1**). Collectively, these diverse projection neuron subtypes account for nearly all the excitatory neurons in the adult neocortex, roughly 85% of total neocortical neurons (Gord and Bernardo, 2011).

One broad class of neocortical projection neurons, called corticofugal projection neurons (CFuPN; *fugal ~ flight*; Latin), constitutes the primary output neuron population of the neocortex. Distinct types and subtypes of these CFuPN are intermixed in the deep layers across all neocortical areas and have axons that exit the neocortex and selectively innervate distant targets in the central nervous system, including thalamus, striatum, brainstem, and spinal cord. Corticospinal motor neurons (CSMN) are one clinically important corticofugal subtype; they reside in motor cortex layer V, and project axons to the spinal cord to control motor function. CSMN specifically degenerate in amyotrophic lateral sclerosis (ALS / ”Lou

Gehrig's disease"), and CSMN axonal damage after spinal cord injury (SCI) is central to ensuing loss of motor function.

Because there are no effective therapies for CSMN-specific and related CFuPN dysfunction, and because sufficient quantities of these neurons are not readily available for *in vitro* disease modeling and development of new treatments, I explored multiple approaches to generate these neurons from mouse embryonic stem (ES) cells. Although pluripotent ES cells have theoretical potential to generate large quantities of CFuPN *in vitro*, existing ES cell protocols are not directed toward generating specific classes, types, or subtypes of neocortical projection neurons (Hansen *et al.*, 2011).

In recent years, tremendous progress has been made to understand stepwise developmental molecular programs of distinct neocortical projection neuron classes, types, and subtypes *in vivo* (Molyneaux *et al.*, 2007; Fame *et al.*, 2011; Woodworth *et al.*, 2012; MacDonald *et al.*, in press; Custo Greig *et al.*, in editorial revision, 2013). Importantly, the application of these advances to CFuPN-directed differentiation of ES cells forms the basis for all approaches and experimental findings discussed in my dissertation. This introductory chapter serves to delineate an emerging molecular understanding of CFuPN development, to provide perspectives on the development and limitations of existing protocols of ES cell directed differentiation, and, lastly, to broadly introduce my approaches to apply CFuPN developmental biology to the directed differentiation of mouse ES cells.

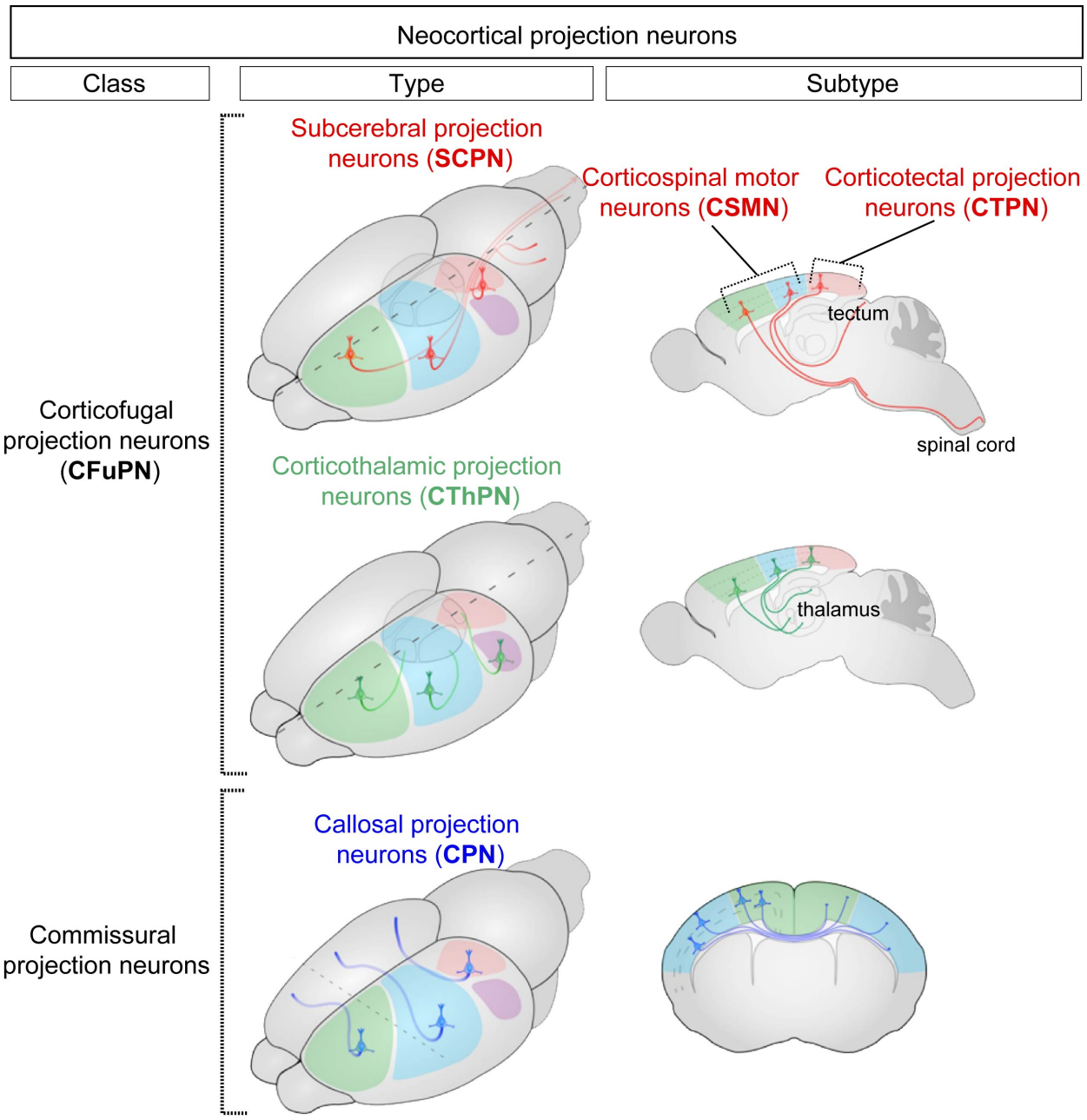

Figure 1.1. Classification of diverse neocortical projection neurons. Neocortical projection neurons can be subdivided into broad classes, types, and subtypes largely based on their axonal projections. Figure adapted from Custo Greig and colleagues (commissioned *Nat Rev Neurosci* manuscript in editorial revision, 2013). Illustrations are of the mouse brain.

1.2. Overview of sequential progenitor and post-mitotic CFuPN development

During development of the neocortex, many diverse projection neuron subtypes are generated under complex and seemingly precise regulation of cell-extrinsic and cell-intrinsic controls. Cell-extrinsic influences initially predominate during early development of the central nervous system (CNS), beginning with blastocyst formation at approximately three days following fertilization, in mice, and lasting until the start of neocortical neurogenesis approximately seven days later. Distinct extracellular molecules regulate the induction of both neural identity and region-specific patterning in the developing nervous system. Importantly, this cell-extrinsic patterning is executed and stabilized by cell-intrinsic transcriptional changes in order to molecularly delineate regions of the nervous system. Later, these cell-intrinsic transcriptional changes become disproportionately important during the transition to neocortical differentiation, and sequentially control the specification of distinct neocortical subtypes. Importantly, stage-specific functions of transcriptional regulators are "nested" within the emerging classes, types, and subtypes of projection neurons during neocortical differentiation (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). Replicating features of this process will be important for directed specification and differentiation of neocortical projection neurons, particularly CFuPN. These multiple cell-extrinsic and cell-intrinsic controls over neocortical projection neuron differentiation will be reviewed and discussed from the perspective of pluripotent stem cell differentiation.

Neural induction: the "default" specification of all neural progenitors

At the earliest stages of mouse embryonic development, approximately embryonic day 3.5 (E3.5), the pluripotent inner cell mass of blastocysts undergoes a process of "neural induction" to delineate the prospective progenitors of the entire CNS (**Figure 1.2**). Induction of

neural progenitor fate occurs largely by preventing the acquisition of alternative mesoderm and endoderm lineages signaled by distinct types of secreted molecules, collectively called morphogens. In the prospective neural region of the blastocyst, signaling pathways of bone morphogenic proteins (BMPs) must be repressed; this is initially accomplished by fibroblast growth factor (FGF) signaling, and later by chordin and noggin, which are secreted by the "node," a primitive organizing center of the early embryo (Levine and Brivanlou, 2007). Many of these *in vivo* mechanisms of neural induction have been investigated using pluripotent mouse embryonic stem (ES) cells, and are largely replicable *in vitro* (Aubert *et al.*, 2002; Ying *et al.*, 2003; Watanabe *et al.*, 2005; Gaspard and Vanderhaeghen, 2010).

Because the mere inhibition of alternate signaling pathways (*e.g.*, BMP; activin/TGF-beta, Wnt) is sufficient for neural induction, neural fate is seemingly the "default" path of embryonic differentiation (Jessell and Sanes, 2000; Rallu *et al.*, 2002). For this reason, a number of protocols of ES cell differentiation induce neural differentiation by the removal of serum, which contains BMPs; within hours of serum removal, mouse ES cells express neural markers (Smukler *et al.*, 2006). To improve the efficiency of this process in human pluripotent cells, "default" neural differentiation can be supported by BMP pathway inhibition using Noggin and a small molecule inhibitor of SMAD signaling (Chambers *et al.*, 2009). Ultimately, multiple effectors of neural differentiation include transcriptional regulators, particularly Sox2, which reinforces cell-intrinsic neural differentiation (reviewed in Sarkar and Hochedlinger, 2013) by transcriptionally repressing alternate mesoderm and endoderm fates in the developing blastocyst and in human pluripotent stem cells (Keramari *et al.*, 2010; Thomson *et al.*, 2011). These potent transcriptional functions of Sox2 can be similarly exploited in the "reprogramming" of fibroblasts to neural progenitors (Ring *et al.*, 2012; Lujan *et al.*, 2012).

Figure 1.2. Cell-extrinsic and cell-intrinsic factors regulate the development of CFuPN within sequential, "nested" stages of differentiation.
(A) "Default" neural and rostral differentiation occurs by the repression of alternate signaling pathways induced by multiple morphogens (*e.g.*, Noggin inhibits BMP signaling during neural plate formation at ~E3.5-E6.5 in mice; telencephalic progenitors require low or absent expression of caudalizing retinoids (RA) and ventralizing Shh at ~E6.5-E8.5).
(B) The dorsal aspect of the telencephalon is called the pallium, which gives rise to the neocortex. In contrast, the ventral telencephalon is called the subpallium. The delineation of these two telencephalic progenitor domains occurs between ~E8.5 and ~E10.5.
(C) During corticogenesis, beginning at ~E10.5 in mice, multiple diverse neocortical projection neuron classes, types, and subtypes are sequentially generated from neocortical progenitors in the pallium. These projection neurons become refined with continued maturation through post-natal ages.
(D) Early stages of CFuPN differentiation are largely mediated by cell-extrinsic factors, whereas later stages of CFuPN differentiation are largely mediated by cell-intrinsic factors.
(E) Following Shh-mediated dorsal-ventral patterning of the telencephalon, pallial and subpallial identities are reinforced by transcriptional regulation (Pax6 and Sox6 in the pallium; Gsh2 in the subpallium).
(F) Pallial progenitors give rise to neocortical progenitors, which generate projection neuron subtypes during corticogenesis at ~E10.5. Early-born CFuPN populate the deep layers of the neocortex. Later-born CPN populate both deep and superficial layers of the neocortex. The molecular distinction of CPN and CFuPN occurs with continued maturation (represented by the transition from yellow, dual-marker expression to either red or green single-marker expression).
(G) "Nested" expression of distinct transcriptional regulators at distinct developmental stages promotes stepwise CFuPN differentiation.

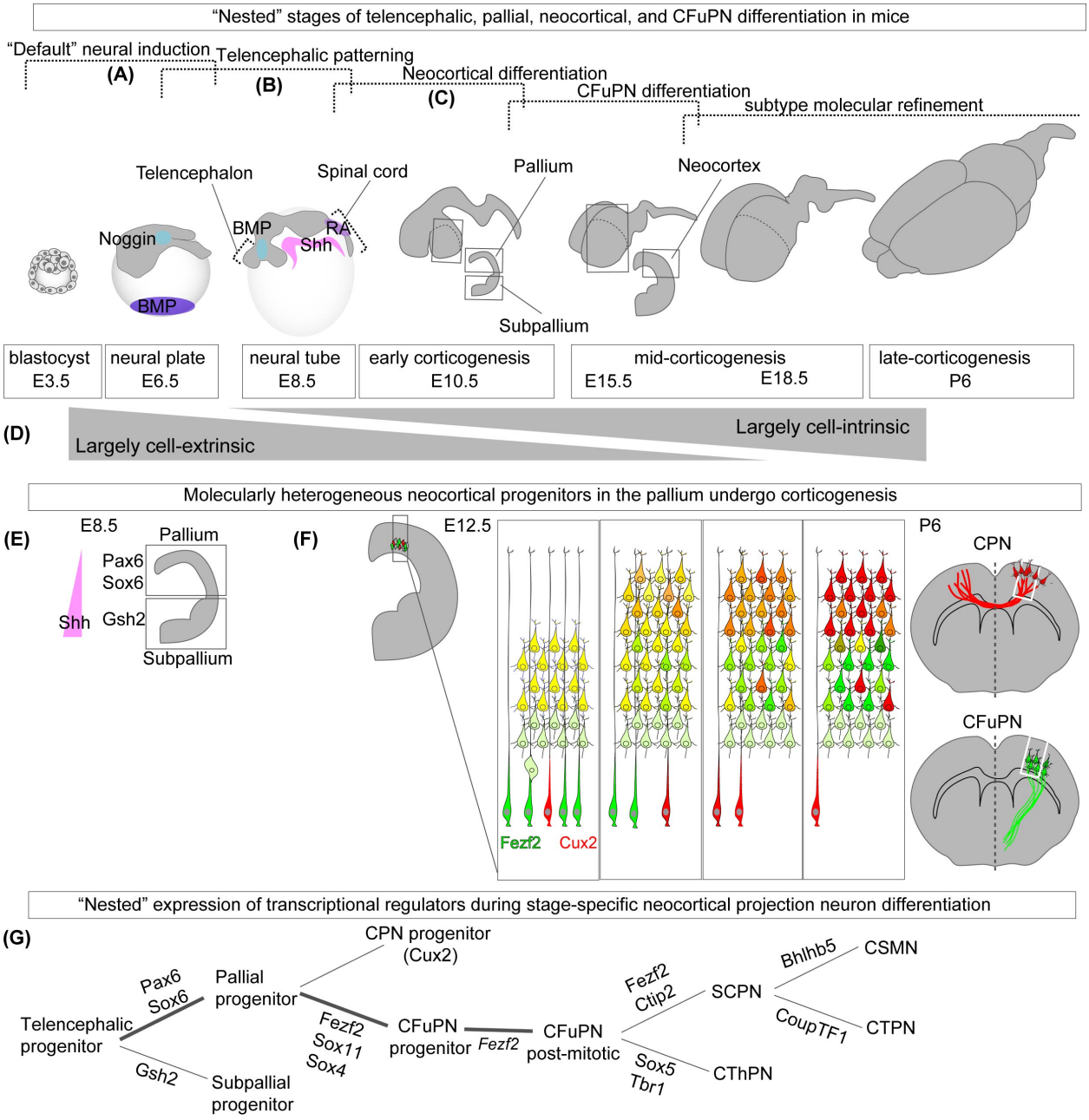

Figure 1.2 (Continued)

Rostral and dorsal regional patterning of neocortical progenitors

Following neural induction, the prospective CNS continues to proliferate, and transitions from a "plate" at E6.5, in mice, to a closed "tube" containing two telencephalic protrusions at the rostral end at E8.5. During the course of these dynamic changes in morphology, distinct morphogen gradients along the rostral-caudal and dorsal-ventral axes pattern the prospective regions of the neural tube (*e.g.*, Wnt and BMPs, rostrally/dorsally; FGFs and retinoids, caudally; Shh ventrally) (Monuki and Walsh, 2001; Rallu *et al.*, 2002; Rash and Grove, 2006). Strikingly, the later roles of some morphogens (*e.g.*, Wnts for rostral/dorsal patterning) greatly differ from their earlier roles in promoting non-neural lineages in the blastocyst. These temporally dynamic changes in Wnt signaling have been leveraged in protocols of ES cell differentiation; for example, Wnt inhibition for neural induction is followed by Wnt activation for subsequent dorsal patterning of ES-derived neural progenitors (Watanabe *et al.*, 2005; Eiraku *et al.*, 2008; Nasu *et al.*, 2012). However, *in vitro* simplifications of a complex extracellular environment can sometimes result in markedly different outcomes of ES cell differentiation; for example, retinoid-independent ES cell differentiation in aggregate culture can generate either predominantly rostral (Watanabe *et al.*, 2005; Eiraku *et al.*, 2008) or predominantly caudal (Peljto *et al.*, 2010; Patani *et al.*, 2011) neural progenitors, depending on the modulation of Wnt signaling. Moreover, because much of this information has been consolidated and derived from studies in frogs and chicks, some of these discrepancies might pertain to species-specific differences (Jessell and Sanes, 2000; Levine and Brivanlou, 2007).

In an analogous situation to "default" neural induction, the rostral specification of neural tube progenitors largely occurs in the absence of caudal-derived, spinal cord morphogens (*e.g.*, FGFs and retinoids). Default rostral differentiation constitutes the first step of most protocols of telencephalic differentiation by ES cells (Gaspard and Vanderhaeghen, 2010; Hansen *et al.*, 2011). Importantly, the identity of rostral progenitor regions is reinforced by the cell-intrinsic

expression of transcriptional regulators, including Otx2, which demarcates the midbrain-hindbrain boundary and is required for early specification of forebrain and midbrain (Acampora *et al.*, 1999). Together, rostral "default" differentiation and transcriptional regulation (*e.g.* Otx2) maintain telencephalic identity.

The dorsal telencephalon, called the *pallium*, ultimately gives rise to all neocortical projection neurons and contributes to the differentiation of other cortical structures, including the cingulate cortex and hippocampus. In contrast, the ventral telencephalon, called the *subpallium*, gives rise to all neocortical interneurons and inhibitory neurons of the basal ganglia, including striatum and globus pallidus (Schuurmans and Guillemot, 2002; Guillemot, 2005; Hébert and Fishell, 2008).

Shh expression gradients (high ventral, low dorsal) are chiefly responsible for the early ~E8.5 delineation of these pallial and subpallial progenitor domains in the telencephalon, occurring prior to neocortical differentiation in the pallium. Specification of the pallium (dorsal telencephalon) occurs in the absence of Shh signaling and in the presence of Wnts and BMPs (Rallu *et al.*, 2002; Tiberi *et al.*, 2012). Shh is secreted from the ventral mesoderm underlying the full extent of the neural tube, and Shh is important for the broad specification of ventral fates in the developing CNS, mediated by Shh repression of Gli family transcription factors. In contrast, in prospective dorsal regions, Gli3 delineates the early pallium in the absence of Shh signaling (Fuccillo *et al.*, 2006; Hoch *et al.*, 2009). Importantly, antagonism of Shh signaling can be exploited in the differentiation of mouse ES cells to dorsal, pallial fates (Gaspard *et al.*, 2008), although this step does not similarly enhance the generation of pallial progenitors by human pluripotent stem cells (Shi *et al.*, 2012; Mariani *et al.*, 2012; Espuny-Camacho *et al.*, 2013). In addition to blocking Shh signaling, pallial-promoting morphogens (*e.g.*, Wnt) have been used for the dorsal patterning of ES-derived neural progenitors (Watanabe *et al.*, 2005;

Eiraku *et al.*, 2008; Nasu *et al.*, 2012). In summary, dorsal patterning of the telencephalon is largely delineated by morphogens and reinforced by transcriptional regulation.

During continued development of the pallium from E8.5-10.5, distinct mechanisms of cross-repressive transcriptional regulation, mediated by Pax6 and Sox6 in the pallium and by Gsh2 in the subpallium, establish the broad molecular identity of these progenitor domains. Pax6 is a well-studied and critical pallium-restricted transcription factor that promotes pallial fate in part by reciprocal and robust cross-repression of Gsh2, whose expression is restricted to the subpallium (Schuurmans and Guillemot, 2002). Importantly, dose-dependent Pax6 expression is required for proper neocortical specification (Schedl *et al.*, 1996; Manuel *et al.*, 2007). Moreover, misexpression of Pax6 in early postnatal cortical astroglia potently directs their conversion to glutamatergic neuronal fates (Berninger *et al.*, 2007; Heins *et al.*, 2002). Complementary to the role of Pax6, a second pallial transcription factor, Sox6, is also required for the proper specification of pallial progenitors; its absence results in misspecification of pallial progenitors, based on the ectopic expression of subpallial genes including Mash1 (Azim *et al.*, 2009). At the same time, multiple other transcriptional regulators (*e.g.*, Sox2 and Lhx2) contribute to increasingly precise pallial progenitor differentiation. In addition to its role at the E3.5 blastocyst stage, Sox2 is required by E8.5 for proper pallial differentiation (Aota *et al.*, 2003; Götz *et al.*, 1998; reviewed in Georgala *et al.*, 2011). Lhx2 is also required for proper neocortical progenitor development by E10.5 (Chou and O'Leary, 2013; Roy *et al.*, 2013). Thus, multiple transcriptional regulators expressed in the pallium (Pax6, Sox6, Lhx2 and Sox2) ultimately enable the proper differentiation of neocortical progenitors by E10.5, which increasingly relies on cell-intrinsic mechanisms of neocortical development.

Potentially CFuPN fate-restricted neocortical progenitors

Beginning at E10.5, a neocortical subdomain of the pallium resolves into a molecularly diverse population of neocortical progenitors that are potentially fate-restricted to distinct projection neuron classes (Franco *et al.*, 2012; Molyneaux *et al.*, 2007; Custo Greig *et al.*, in editorial revision, 2013). One subset of neocortical progenitors is fate-restricted to the broad class of commissural neocortical projection neurons: fate-mapping experiments have decisively shown that Cux2-expressing progenitors exclusively generate callosal projection neurons (CPN) (Franco *et al.*, 2012). In addition, increasing evidence indicates the existence of a second subset of neocortical progenitors that is potentially fate-restricted to subcerebral projection neurons, SCPN, one type of CFuPN that projects to targets caudal to thalamus. This evidence includes heterogeneous, "salt-and-pepper" distribution of Fezf2 expression among pallial progenitors and mutant data confirming the requirement of Fezf2 for specification of SCPN (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Shim *et al.*, 2012). Because Fezf2 is differentially expressed in all CFuPN, one leading hypothesis is that Fezf2-expressing progenitors give rise to the broad class of CFuPN, whose subsequent fate-restriction into distinct corticofugal types and subtypes is later controlled and modified by additional transcriptional regulators that are "nested" within these subtypes. This hypothetical "developmental logic" is consistent with recent findings in the field, and potentially explains the cell-intrinsic control of sequentially emerging classes, types, and subtypes of projection neurons during neocortical differentiation (reviewed in Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013).

Progressive transcriptional refinement of CFuPN identity

During the process known as corticogenesis, neocortical progenitors sequentially generate many diverse classes, types, and subtypes of neocortical projection neurons that

populate the neocortex in an approximately inside-out manner from ~E10.5 until the end of embryonic development ~E18.5/E19.5 (**Figure 1.2**). Strikingly, this sequential generation of broad classes and types of neocortical projection neurons occurs in a largely cell-autonomous manner (Shen *et al.*, 2006), although several cell-extrinsic factors are also important (Hashimoto-Torii *et al.*, 2008; Rodriguez *et al.*, 2012; Diaz-Alonso *et al.*, 2012; Johansson *et al.*, 2013; reviewed in Tiberi *et al.*, 2012).

From ~E10.5 until ~E14.5 in mice, neocortical progenitors give rise to distinct types of Fezf2-expressing CFuPN, including corticothalamic projection neurons (CThPN), and SCPN (**Figure 1.1**). These early-born CFuPN types sequentially populate the deep layers of the neocortex: CThPN predominantly in layer VI and SCPN predominantly in layer V. Initially, these distinct CFuPN types share the expression of multiple transcriptional regulators, but co-expression is then restricted in a subtype-specific manner later in differentiation (Lai *et al.*, 2008; Azim *et al.*, 2009). Overall, multiple transcriptional regulators "nested" within the broad class of CFuPN promote the molecular distinction of SCPN versus CThPN.

SCPN-specific specification, post-mitotic differentiation, axon guidance, and axon pruning are in large part collectively controlled by the transcriptional activities of Fezf2, Ctip2, and Otx1 (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Arlotta *et al.*, 2005; Chen *et al.*, 2008; Shim *et al.*, 2012). Although these three transcription factors are initially expressed in both SCPN and CThPN, the dose and timing of their expression strongly indicates SCPN-specificity. Firstly, Fezf2 is required for the specification of SCPN, but not CThPN. In the absence of Fezf2, downstream Ctip2 transcription factor expression is lost, and prospective SCPN instead differentiate into two populations: commissural neurons, based on the appearance of axonal projections across the corpus callosum and anterior commissure; and CThPN, based on the ectopic expression of Tbr1 in layer V and the abundance of thalamic projections (Molyneaux *et*

al., 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Bedogni *et al.*, 2010; McKenna *et al.*, 2011). Secondly, Ctip2 is critical for the post-mitotic acquisition of SCPN identity, and, in its absence, SCPN do not project to the spinal cord, with additional defects in axon outgrowth, fasciculation, and guidance. Thirdly, Otx1 is specifically expressed in post-mitotic SCPN and directs pruning of SCPN axonal projections during late post-natal maturation (Weimann *et al.*, 1999). Together, Fezf2, Ctip2, and Otx1 are essential for the proper differentiation of SCPN.

In contrast, the distinction of CThPN from later-born SCPN is, in large part, controlled by the interactions of transcriptional regulators that include Sox5 and Tbr1. Although these two transcription factors are initially expressed in both SCPN and CThPN, the dose and timing of their expression strongly indicates CThPN-specificity, where these factors repress Fezf2 expression and SCPN identity (Lai *et al.*, 2008; Shim *et al.*, 2012; Bedogni *et al.*, 2010; McKenna *et al.*, 2011). In particular, Sox5 is expressed by two forebrain populations, neocortical post-mitotic CFuPN and subpallial progenitors (Lai *et al.*, 2008). The deletion of Sox5 in CFuPN blurs the temporally distinct and sequential generation of CThPN followed by SCPN: some prospective CThPN that are Sox5-deficient alternately acquire SCPN-like axonal projections (Lai *et al.*, 2008). Together, multiple type-specific transcriptional refinements (*e.g.*, Fezf2/Ctip2 versus Sox5/Tbr1) contribute to the corticofugal class-specific distinction of CThPN and SCPN.

Leveraging an emerging logic of CFuPN differentiation

The overall biology of transcriptional regulation that is “nested” within distinct and increasingly fate-restricted classes, types, and subtypes during maturation is not limited to type-specific distinction of CThPN and SCPN within the class of CFuPN. In fact, inter-related processes of transcriptional refinements are shared by nearly all projection neuron classes,

including commissural projection neurons, and by subtypes distributed in multiple neocortical areas (Lai *et al.*, 2008; Britanova *et al.*, 2008; Alcamo *et al.*, 2008; Chen *et al.*, 2008; Azim *et al.*, 2009; Joshi *et al.*, 2008; Cederquist *et al.*, 2013). An emerging model of these molecular refinements encompasses an "n-dimensional subtype space" in which subtype distinction occurs on multiple axes of neocortical projection neuron identity (*e.g.*, areas, layers, and hodological subtypes); this subtype-distinction is largely guided by the "nested" expression of distinct transcriptional regulators within these diverse subtypes (Custo Greig *et al.*, in editorial revision, 2013).

Pertinent to my approaches of directed differentiation of CFuPN, this emerging framework of neocortical development delineates distinct roles for transcriptional regulators that act at transient stages of differentiation, in specific combinations, at specific doses, and within specific classes of projection neurons. Because sequential transcriptional refinements appear essential to the precise specification of neocortical projection neuron subtypes, this transcriptional logic of neocortical differentiation forms the basis for my approaches to directed differentiation of ES cells into CFuPN.

1.3. Application of telencephalic development to ES cell differentiation

Embryonic stem cells have potential to generate CFuPN

Mouse embryonic stem (ES) cells were first derived from the inner cell mass of blastocysts in 1981 (Evans and Kaufman, 1981; Martin, 1981). Because pluripotency is a normally transient embryonic state, ES cells immediately differentiate into more restricted cell lineages. However, in 1988, Austin Smith and colleagues established the first *in vitro* conditions for the stable, long-term maintenance of mouse ES cell pluripotency by the identification of leukemia inhibitory factor (Smith *et al.*, 1988). This discovery was later followed by the derivation of human ES cells (Thomson *et al.*, 1998), which are optimally cultured in the presence of bFGF to maintain their long-term pluripotency. Interestingly, the difference between mouse and human ES cell pluripotency requirements is largely attributable to the timing of derivation; mouse ES cells resemble an early, "ground" state of pluripotency at the blastocyst stage ~E3.5 (Ying *et al.*, 2008), whereas human ES cells resemble a later, "primed" state of pluripotency derived from the late blastocyst (epiblast) stage ~E4.5 (Nichols and Smith, 2009).

Since their derivation, mouse ES cells have been touted for their proven ability to generate all tissue and cell types *in vivo*, and their theoretical ability to generate this repertoire of cell types *in vitro* (Smith, 2001). Given the vast potential of ES cells to acquire diverse cellular identities, Waddington's 'epigenetic landscape' is a useful model for visualizing potential stochastic, cell-intrinsic, and cell-extrinsic mechanisms of sequential cell fate determination (Waddington, 1957; reviewed in Hochedlinger and Plath, 2009) (**Figure 1.3**). In the Waddington model, a ball rolling down a hilly landscape represents the trajectory and progressive restriction of cell fate determination. From this perspective of differentiation, a number of groups have successfully "directed" differentiation of ES cells along specific

trajectories of cell fate determination, by taking advantage of a combination of early cell-extrinsic patterning (*e.g.*, regional specification of the neural tube) followed by spontaneous subtype differentiation (Wichterle *et al.*, 2002; Watanabe *et al.*, 2005; Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Nasu *et al.*, 2012; Espuny-Camacho *et al.*, 2013).

Strategies of morphogen-based "directed" differentiation

The first successful example of "directed," region-specific neuronal differentiation from ES cells (Wichterle *et al.*, 2002) leveraged an understanding of two important aspects of *in vivo* spinal cord development: 1) distinct morphogen gradients along the rostral-caudal (*e.g.*, retinoids) and dorsal-ventral (*e.g.*, Shh) axes of the neural tube; 2) transcriptional profiling of spinal cord progenitor domains and post-mitotic neurons (Jessell, 2000; Briscoe *et al.*, 2000; Shirasaki and Pfaff, 2002), including the identification of Hb9, an exclusive marker of spinal motor neurons (SMN) (Arber *et al.*, 1999; Thaler *et al.*, 1999). With this information, Wichterle and colleagues in the Jessell lab hypothesized that distinct concentrations of known morphogens could grossly induce caudal (spinal cord) and ventral (motor neuron) progenitor specification by ES cells in three steps, using previously-established protocols of embryoid body ES cell culture: 1) "default" rostral neural induction by removal of serum/BMP; 2) caudalization by retinoids; and 3) ventralization by Shh (Wichterle *et al.*, 2002). Retinoid- and Shh-patterned, ES-derived ventral spinal cord progenitors spontaneously give rise to a small, molecularly heterogeneous population of neurons that include Hb9-expressing SMN in free-floating embryoid body cell culture. This small population of ES-derived SMN has multiple characteristics of *in vivo* SMN axonal connectivity and physiology, particularly after intra-spinal transplantation in the developing chick (Wichterle *et al.*, 2002; Peljto *et al.*, 2010) or co-culture with chick myotubes (Miles *et al.*, 2004).

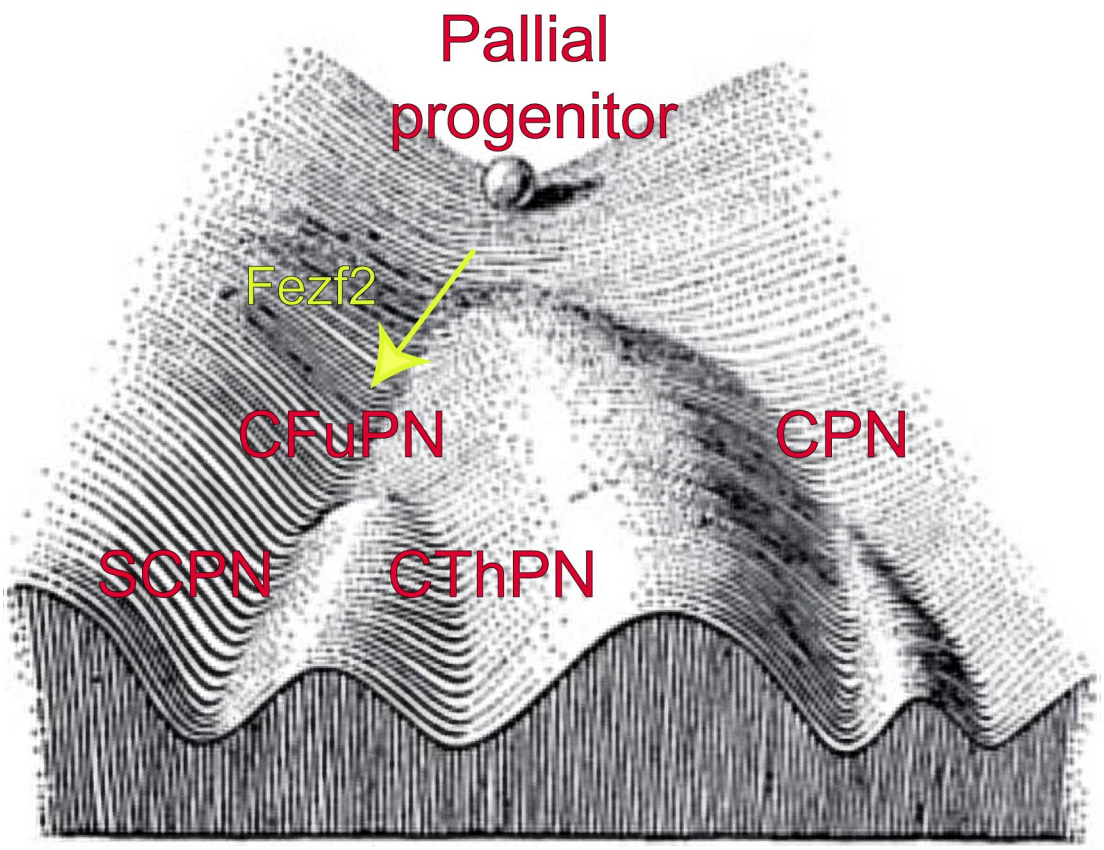

Figure 1.3. The Waddington "epigenetic landscape" model depicts combined cell-extrinsic, cell-intrinsic, and stochastic influences during differentiation, consistent with sequential, "nested" neocortical projection neuron development. Stepwise CFuPN development is consistent with the Waddington model of progressive fate restriction (reviewed in Hochedlinger and Plath, 2009) and contrasts with the hypothesis that single "transcriptional codes" might define and directly reprogram distinct neuronal subtypes (Ladewig *et al.*, 2013). Fezf2 is a potent transcription factor that is specifically expressed by CFuPN in the neocortex, and is required for the specification of SCPN. Multiple lines of evidence suggest that Fezf2 cannot directly promote SCPN identity; its actions are likely context-dependent (represented by the localized arrow at a bifurcation point in the selection of CFuPN versus CPN identity). This figure has been adapted from C. H. Waddington (1957).

Multiple groups have adapted morphogen-based strategies to promote the regional specification of rostal and dorsal telencephalic progenitors by ES cells (Hansen *et al.*, 2011). Similar to the example of ES-derived SMN, the first protocol of "directed" telencephalic regional differentiation from ES cells (Watanabe *et al.*, 2005) used an understanding of *in vivo* telencephalic development, principally involving "default" rostral neural induction by removal of serum/BMP and direct Wnt and Nodal antagonism followed by Wnt activation. Telencephalic progenitors are identifiable by expression of an early telencephalon-restricted transcription factor, Foxg1 (Tao and Lai, 1992; Xuan *et al.*, 1995). Building from this foundation, and using recently identified transcriptional controls over neocortical projection neuron differentiation to label *in vitro* neuronal populations (*e.g.*, Ctip2, Arlotta *et al.*, 2005; Satb2, Alcamo *et al.*, 2008; Britanova *et al.*, 2008; reviewed in Molyneaux *et al.*, 2007), Eiraku and colleagues in the Sasai lab generated neocortical-like neurons within self-organized telencephalic embryoid body culture (Eiraku *et al.*, 2008; Nasu *et al.*, 2012; detailed protocol in Eiraku and Sasai, 2012). Interestingly, this embryoid body culture recapitulates some aspects of three-dimensional neocortical progenitor and neuron lamination, with heterogeneous neocortical neuron marker expression, grossly resembling early telencephalic differentiation.

Development of monolayer protocols for neocortical-like differentiation

Monolayer ES cell culture has multiple advantages over embryoid body culture that include easy visualization and access during the course of *in vitro* differentiation. Monolayer culture has recently been successfully applied to the regional specification of pallial-like progenitors by ES cells (Gaspard *et al.*, 2008; Tiberi *et al.*, 2012; Espuny-Camacho *et al.*, 2013). The many advantages of monolayer culture have, for example, enabled the identification of largely intrinsic neocortical differentiation by individual progenitors isolated from E10.5 pallium

(Shen *et al.*, 2006; reviewed in Okano and Temple, 2009). One of the first successful monolayer protocols of ES cell neural induction identified specific culture conditions that included the removal of serum and LIF (Ying *et al.*, 2003).

Building on these advances, Gaspard and colleagues in the Vanderhaeghen lab defined monolayer culture conditions of pallial-like progenitor specification by ES cells (Gaspard *et al.*, 2008; detailed protocol in Gaspard *et al.*, 2009). This protocol was aided by the prior development of a protocol promoting telencephalic ES cell differentiation (Watanabe *et al.*, 2005) and by the recent identification of post-mitotic markers of distinct classes of neocortical projection neurons (*e.g.*, Ctip2 and Satb2). The protocol by Gaspard and colleagues (2008, 2009) makes use of "default" rostral differentiation, with the additional step of Shh antagonism by cyclopamine, to generate largely rostral and dorsal pallial-like differentiation. These ES-derived pallial-like progenitors spontaneously generate heterogeneous neuronal populations that express individual characteristics of neocortical-like neurons, as well as the gross temporal order of marker expression in the developing neocortex. Considered a model of *in vitro* corticogenesis, this approach is now being successfully applied to *in vitro* investigations of neocortical biology by these ES-derived populations (Tiberi *et al.*, 2012). Notably, adaptations of this monolayer protocol to human pluripotent stem cell culture have distinct differences in comparison to mouse ES cell culture: first, Shh antagonism is not necessary (Espuny-Camacho *et al.*, 2013); second, pallial-like progenitor differentiation is enhanced by the late addition of retinoids (Shi *et al.*, 2012; Mariani *et al.*, 2012). This approach of monolayer ES cell differentiation is the starting point for my experimental approaches in this dissertation.

Toward CFuPN-specific differentiation

While the well-established monolayer protocol of ES cell differentiation is a substantial technical advance toward the directed differentiation of neocortical fates, the pallial-like

progenitors and neocortical-like neurons have not been assessed for their expression of stage-specific transcriptional regulators. Moreover, because of its reliance on spontaneous differentiation, this protocol has not been directed toward differentiation of specific classes, types, or subtypes of neocortical projection neurons.

To extend the principles of morphogen-directed differentiation from the generation of heterogeneous neocortical-like neurons to the specific generation of CFuPN, my approaches in this dissertation are largely based on the emerging understanding of neocortical projection neuron development (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). This framework of neocortical development delineates distinct roles for transcriptional regulators that act in stage-specific combinations, at specific doses, and especially within specific classes of projection neurons. Moreover, stepwise transcriptional regulation is consistent with the Waddington epigenetic landscape model of progressive fate restriction (Hochedlinger and Plath, 2009) and contrasts with the hypothesis that single "transcriptional codes" might define and directly reprogram distinct neuronal subtypes (Ladewig *et al.*, 2013). Additional evidence against a "transcriptional code" mechanism of neocortical development includes the argument that none of approximately 2,000 transcription factors expressed by mammalian genomes are specific to single neuronal subtypes in the nervous system. In fact, many transcriptional regulators described in this dissertation have critical roles in hematopoiesis, further supporting the exquisite context dependence of transcriptional regulation in neocortical differentiation. Overall, sequential transcriptional refinements appear essential to the specification of neocortical projection neuron subtypes, and this developmental framework forms the basis for my approaches to directed differentiation.

1.4 Dissertation overview

In this dissertation, I introduce multiple approaches that apply an emerging understanding of neocortical and CFuPN development to both evaluate and direct mouse ES cell differentiation to CFuPN, the output neurons of the neocortex. Although ES cells have theoretical potential to generate CFuPN *in vitro*, existing ES cell differentiation protocols are not directed toward specific classes, types, or subtypes of neocortical projection neurons and instead rely on the spontaneous generation of neurons with neocortical-like characteristics.

In Chapter 2, I first successfully replicate an established monolayer protocol of dorsal telencephalic differentiation that generates neurons with overall characteristics of neocortical differentiation (Gaspard *et al.*, 2009). Using this protocol, I present experimental results strongly indicating that these spontaneously-generated, ES-derived neocortical-like neurons are heterogeneous, immature, and "stalled" at a stage roughly equivalent to mid-embryonic differentiation *in vivo*.

In Chapter 3, I build on that foundation to promote CFuPN-specific differentiation among these "stalled" neocortical-like neurons using a combination of recently developed synthetic modified mRNA (modRNA) and high-content chemical screening technologies. These strategies address potential chromatin remodeling deficits during the spontaneous differentiation of neocortical-like neurons by ES cells.

In Chapter 4, I take advantage of the framework of "nested" transcriptional regulation driving neocortical projection neuron differentiation, by sequentially expressing critical forebrain, neocortex, and CFuPN-specific transcription factors to direct differentiation of ES cells into CFuPN. When micro-transplanted into neonatal neocortex, CFuPN-directed ES-derived neurons appropriately and relatively specifically innervate corticofugal targets in the thalamus and midbrain, in contrast to neurons generated by prior methods. Overall, my

experiments rigorously characterize and then successfully enhance the directed differentiation of mouse ES cells into CFuPN.

Finally, in Chapter 5, I critically review my experimental findings and discuss topics of directed differentiation that have broad relevance to the field, including: 1) commonalities of maturation defects in many *in vitro* protocols of directed differentiation, potentially owing to "confusion" of molecular identity; 2) potential mechanisms causing immature or "confused" *in vitro* differentiation; and 3) future approaches for directed differentiation of clinically important subtypes (*e.g.* CSMN) within the broad class of CFuPN. Together, these topics delineate the broader challenges and long-term applications of my work for modeling CFuPN-specific neurodegenerative diseases and for developing new treatments for ALS and spinal cord injury.

Chapter 2

An established monolayer protocol for differentiation of mouse embryonic stem cells generates heterogeneous, neocortical-like neurons that are stalled at a stage equivalent to mid-corticogenesis

Publication

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Author contributions

I independently initiated this project, and performed all experimental work. Hari Padmanabhan contributed critically to discussions about the data. Approximately half of the data were acquired in Lee Rubin’s laboratory, in a collaborative use of resources and training. Kelly M. Haston contributed to numerous discussions, and undergraduates Chris Goldstein and Matthew Abrams provided important technical help with specific experiments. I received additional technical support from Amanda W. Gee, Monica Hayhurst, and Lida Katsimpardi.

2.1 Abstract

Neocortical projection neurons arise from discrete progenitor domains in the pallium, where the actions of specific molecular controls progressively refine their differentiation. Two existing and widely applied protocols of embryonic stem (ES) cell differentiation have been developed to enable *in vitro* generation of neurons resembling neocortical projection neurons in monolayer culture (Gaspard *et al.*, 2008; Espuny-Camacho *et al.* 2013) and from embryoid bodies (Eiraku *et al.*, 2008; Nasu *et al.*, 2012); the monolayer approach offers advantages for detailed *in vitro* characterizations and potential mechanistic and therapeutic screening.

I asked whether mouse ES cells undergoing largely undirected neocortical differentiation in monolayer culture recapitulate progressive developmental programs of *in vivo* progenitor and post-mitotic differentiation, and whether they develop into specific neocortical subtypes, using rigorous criteria. I find that ES-derived mitotic cells that have been dorsalized by the Sonic hedgehog antagonist cyclopamine, and that express, as a total population, cardinal markers of telencephalic progenitors, are, in fact, molecularly heterogeneous. This finding is based on the largely non-overlapping expression of critical transcriptional controls over pallial progenitors, including Pax6 and Sox6. The absence of either of these transcription factors *in vivo* results in distinct deficits in neocortical differentiation. I next show that these progenitors subsequently generate small numbers of heterogeneous neocortical-like neurons that are “stalled” at an immature stage of differentiation, based on multiple developmental criteria: 1) sparse expression of mature neuronal markers (MAP2, NeuN); 2) immature and unresolved co-expression of molecular controls over multiple distinct classes and types of neocortical projection neurons; and 3) immature and unresolved expression of critical post-mitotic transcription factors that progressively refine the neocortical area-specific differentiation of projection neuron subtypes.

While some aspects of neocortical development are recapitulated by existing protocols of ES cell differentiation, these data indicate that mouse ES-derived neocortical progenitors are both more heterogeneous than their *in vivo* counterparts, and seemingly include many incorrectly specified progenitors. Further, these ES-derived progenitors spontaneously differentiate into sparse, incompletely and largely imprecisely differentiated, neocortical-like neurons that fail to adopt specific neuronal identities *in vitro*. These results provide both foundation and motivation for refining and enhancing directed differentiation of clinically important neocortical projection neuron subtypes.

2.2 Introduction

Neocortical neurons undergo distinct molecular refinements at progenitor (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Azim *et al.*, 2009) and post-mitotic (Weimann *et al.*, 1999; Arlotta *et al.*, 2005; Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Lai *et al.*, 2008; Joshi *et al.*, 2008; Azim *et al.*, 2009; Tomassy *et al.*, 2010; Cederquist *et al.*, 2013) stages of development. These molecular refinements individually represent distinct developmental programs that, in sequential combinations, control neocortical development. In the absence of these critical transcription factors that control any of these stages, the precise molecular identity, laminar/area positioning, and/or projection patterns of neocortical projection neuron subtypes are disrupted *in vivo*. These transcriptional controls, therefore, are good candidates for rigorous characterization of *in vitro* neocortical-like neurons derived from embryonic stem (ES) cells.

Recent advances in mouse ES cell directed neocortical differentiation recapitulate some, but not all, aspects of corticogenesis (Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Nasu *et al.*, 2012; Hansen *et al.*, 2011). Importantly, populations of ES-derived neocortical-like neurons sequentially express single genes characteristic of neocortical neurons *in vivo*. However, many of these genes (*e.g.*, Pax6, Ctip2, Satb2) are not specific only to the neocortex, but are expressed in other regions of the developing neural tube. For example, Pax6 is differentially expressed throughout the rostro-caudal extent of the neural tube ventricular zone (Ericson *et al.*, 1997; Osumi *et al.*, 1997; Briscoe *et al.*, 2000; Alaynick *et al.*, 2011), and Ctip2 is also expressed in striatum, olfactory bulb, and hippocampus (Leid *et al.*, 2004; Arlotta *et al.*, 2005; Arlotta *et al.*, 2008).

With deeper analysis, using multiple markers, it is increasingly apparent that ES-derived neocortical-like neurons are incompletely specified *in vitro*. First, a substantial fraction of these

neurons express combinations of molecular markers that are not described in the neocortex *in vivo* (*e.g.*, Reelin/Ctip2; Gaspard *et al.*, 2008). Second, ES-derived neocortical neurons often display mixed subtype-specific molecular characteristics, such as co-expression of deep- and superficial-layer markers in individual hES-derived neurons (Mariani *et al.*, 2012; Shi *et al.*, 2012. Finally, these neurons display skewed areal specification and projection patterns to visual and limbic targets (Gaspard *et al.*, 2008; Espuny-Camacho *et al.*, 2013). These subtle but distinct deficiencies in the differentiation of ES-derived neocortical neurons suggest incomplete differentiation, which might hinder neocortical subtype acquisition, and limit the interpretability of these *in vitro* models of corticogenesis.

More refined characterizations of *in vitro* neocortical differentiation are now possible, given recent advances in the study of neocortical development (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). Pax6, often used to exclusively mark the pallium, is not a specific marker of the pallial tissue, given its expression throughout the neural tube (Alaynick *et al.*, 2011). In the absence of positional information *in vitro*, characterization of Pax6-expressing “pallial” progenitors is incomplete without the presence of additional markers of pallial progenitors (*e.g.*, Sox6; Azim *et al.*, 2009; Otx2, Acampora *et al.*, 1999), or the absence of other markers co-expressed with Pax6 outside of the pallium.

Sox6 is a transcription factor that controls the development of pallial progenitors independently from Pax6; its absence results in misspecification of pallial progenitors, by ectopic expression of subpallial genes (Azim *et al.*, 2009). Like Pax6, Sox6 is not specific to the pallium, as it is also expressed by post-mitotic, subpallium-derived interneurons. However, when Sox6 is assessed in combination with Pax6, the presence of both markers greatly

increases the specificity for pallial progenitors. To date, this combination has not been used for the identification of pallial progenitors *in vitro*.

Post-mitotic neocortical neurons *in vivo* undergo a prolonged maturation process, during which gene expression becomes progressively restricted to particular subtypes (Lai *et al.*, 2008; Britanova *et al.*, 2008; Alcamo *et al.*, 2008; Chen *et al.*, 2008; Azim *et al.*, 2009; Joshi *et al.*, 2008; Woodworth *et al.*, 2012; Cederquist *et al.*, 2013; Custo Greig *et al.*, in editorial revision, 2013). These neurons initially co-express transcriptional controls characteristic of multiple neocortical projection subtypes (*e.g.*, Tbr1, Ctip2, Satb2, Ctip1) and multiple neocortical area identities (*e.g.*, CoupTF1, Bhlhb5, Ctip1) before distinct subtype identities emerge. Together, this process of molecular refinement involves, at minimum, coordinated neuronal maturation, neocortical projection neuron class distinction, and neocortical area subtype distinction. These three stage-specific features of neocortical identity refinement form the basis for my approach to characterizing neocortical identity *in vitro*.

I assessed mouse ES cell-derived neocortical-like neurons at progenitor and post-mitotic stages, and identified multiple characteristics consistent with stalled maturation. First, ES-derived neocortical-like progenitors are more heterogeneous than has been previously reported using single-marker analyses. Second, neocortical-like neurons are stalled at a maturation stage resembling mid-corticogenesis, as indicated by overlapping expression of multiple subtype-specific markers that do not resolve with time. Additionally, area-specific differentiation is abnormal, as ES-derived neocortical neurons are deficient in the rostral regulator of neocortical development, Bhlhb5. Overall, this approach rigorously investigates the refinement of ES-derived neocortical differentiation and indicates directions for refining directed differentiation of clinically important neocortical projection neurons.

2.3 Results

To begin characterizing ES-derived neocortical-like cells, I cultured mouse ES cells, and directed their differentiation to neocortical fates using an established monolayer cell culture protocol (Gaspard *et al.*, 2009). This protocol enables rostral and dorsal differentiation by plating ES cells at low-density, removing serum and retinoids, and antagonizing residual Shh morphogen signaling with cyclopamine. I replicated this protocol, and generated sequential waves of broad neural populations (neural progenitors, immature neurons, and astroglia) from E14Tg2a mouse ES cells over the course of 28 days (**Figure 2.1**). After two weeks in culture, approximately 60% of ES-derived cells express Nestin, an intermediate filament protein, broadly marking neural progenitors. Similar results were obtained using Nagy G4 mouse ES cells (data not shown). These results show that both the timing of neural induction, and sequential generation of neural progenitors, neurons, and astroglia, are nearly identical to previously published results (Gaspard *et al.*, 2008).

Distinct subsets of pallial progenitors are generated from ES cells

I first assessed the proportion of rostral, dorsal, pallial-like differentiation by ES-derived Nestin-expressing neural progenitors at *in vitro* day 14. Approximately half of Nestin-expressing progenitors are pallial-like, based on co-expression of Pax6 (**Figure 2.2A**). All Pax6-expressing cells co-express Nestin, and Pax6 is not expressed by any TuJ1 (beta tubulin III)-expressing immature neurons (data not shown). Together, these data suggest that Pax6 expression is restricted to about half of ES-derived neural progenitors.

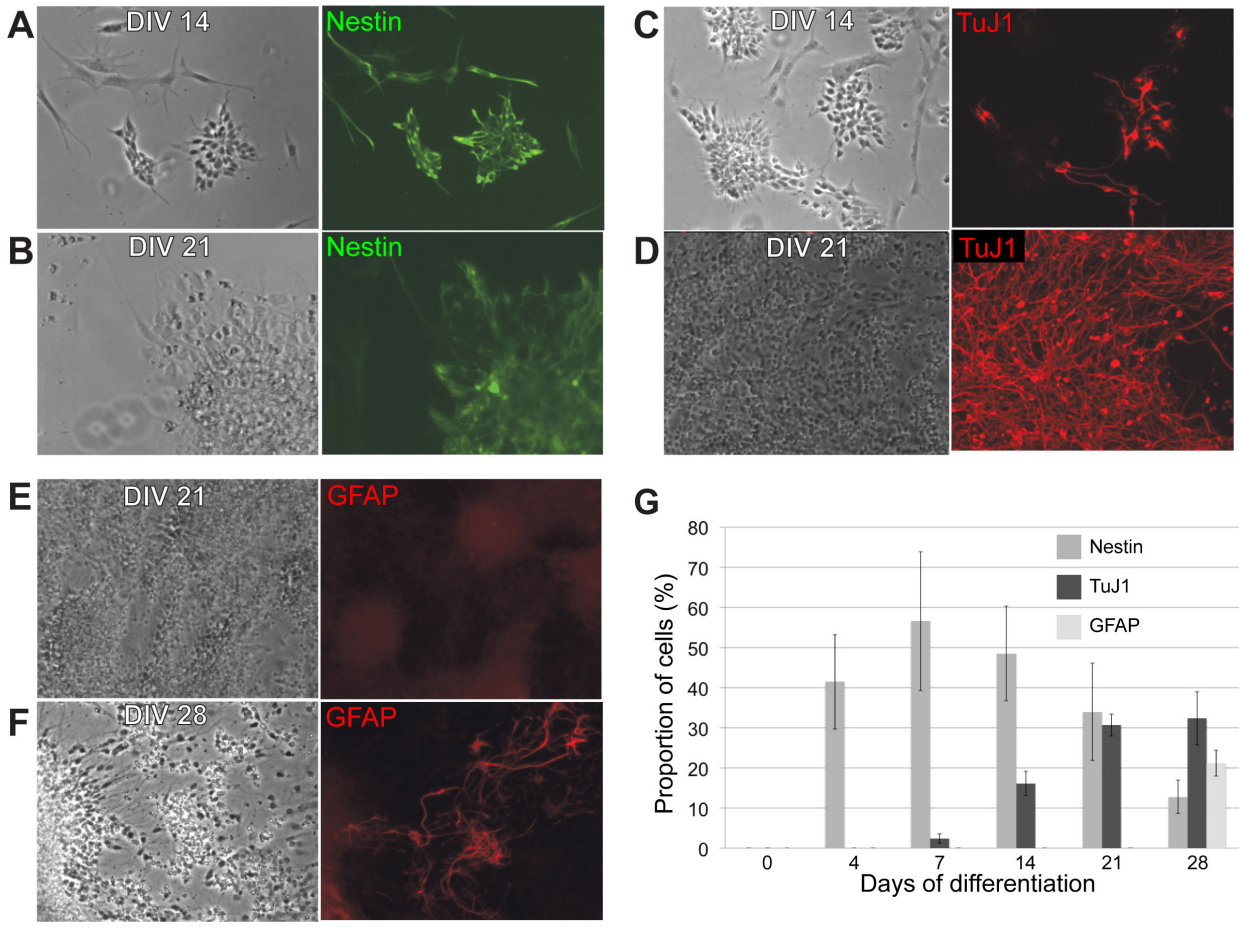

Figure 2.1. Sequential generation of neural progenitors, neurons, and astroglia in an established monolayer ES cell protocol is reproducible. (A, B) Nestin expression decreases, as a proportion of total cells, from day *in vitro* (DIV) 14 to DIV 21. (C, D) TuJ1 expression increases, as a proportion of total cells, from day 14 to day 21. (E, F) GFAP expression begins by day 28. (G) Quantification of Nestin, TuJ1, GFAP expression over the course of 28 days. Data are represented as mean +/- s.e.m. (N = 3).

Figure 2.2. Pallial-like progenitors generated by ES-derived progenitors are molecularly heterogeneous. (A, B) Half of Nestin-expressing progenitors co-express Pax6 (A) or Sox6 (B). (C) ~20% of progenitors express both Pax6 and Sox6. (D, E) The majority of Pax6 (D) and Sox6 (E) pallial progenitors co-express Otx2. (F) Ngn2 is expressed in some Pax6 low/negative progenitors. (G) Mash1/Nestin subpallial-like progenitors represent ~10% of cells. (H-J) Ventralized ES cells lose Pax6 expression (H), and increase subpallial Nkx2.1 (I) and Gsh2 (J). (K, L) Pax6/Mash1 (K) and Sox6/Mash1 (L) cellular subsets are mostly distinct.

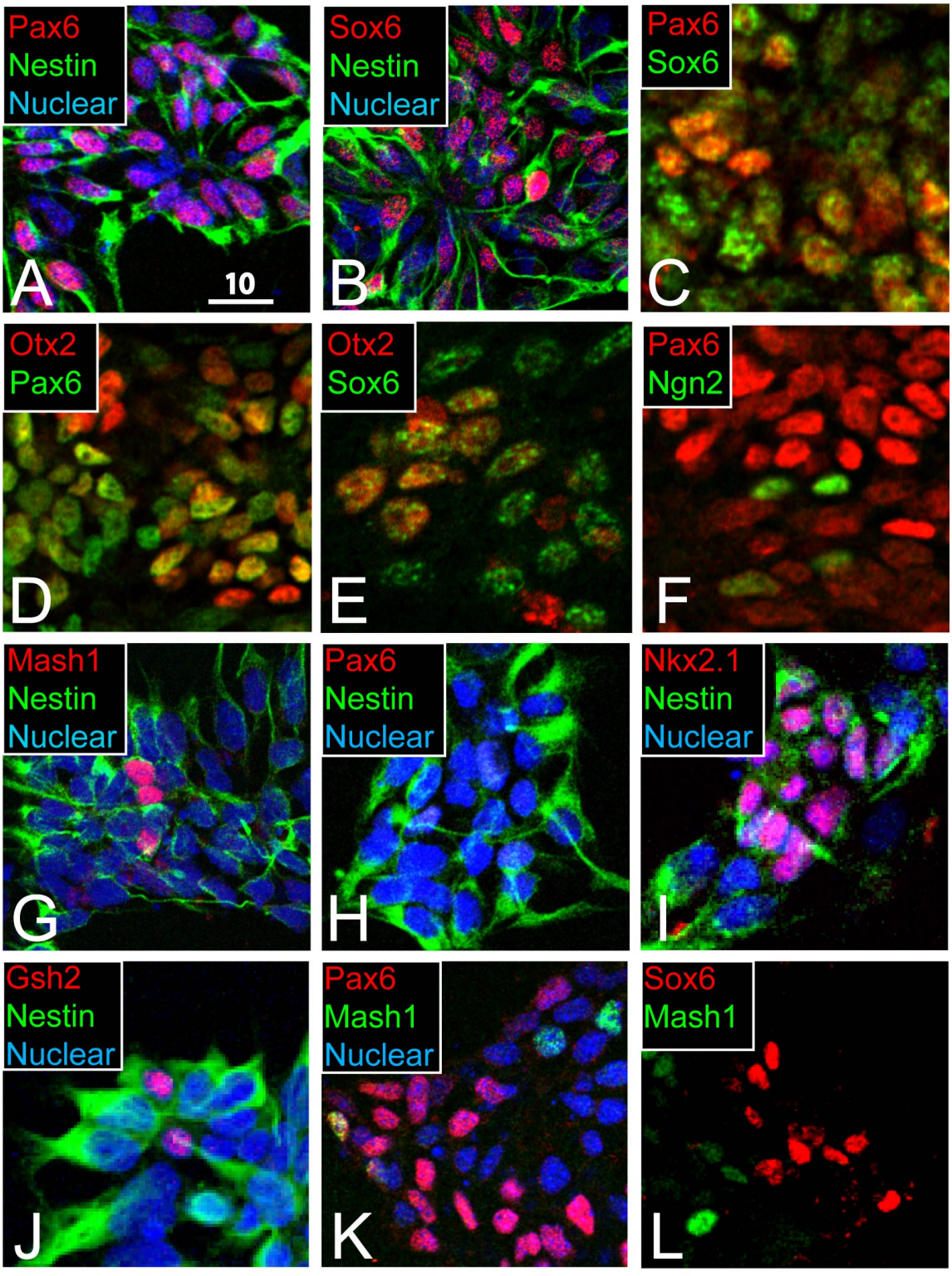

Figure 2.2 (Continued)

I next asked whether these Pax6-expressing neural progenitors at *in vitro* day 14 display other characteristics of pallial progenitors. I hypothesized that correctly-specified pallial progenitors will co-express Sox6 (Azim *et al.*, 2009). Similar to the proportion of Pax6-expressing progenitors, I find that approximately half of Nestin-expressing progenitors also express Sox6 (**Figure 2.2B**). However, Pax6 and Sox6 are co-expressed by only approximately 20% of progenitors (**Figure 2.2C**), which is strikingly dissimilar to their highly overlapping expression *in vivo* (Azim *et al.*, 2009). Overall, the combined distribution of Pax6 and Sox6 expression accounts for the majority of Nestin-expressing progenitors at day 14, but these pallial transcription factor controls are largely not expressed by the same cells.

Since most Pax6-expressing cells do not co-express Sox6, I hypothesized that some Pax6-expressing cells might possess identities characteristic of a position in the neural tube caudal to the telencephalon. Otx2, expressed throughout the ventricular zone of the neural tube rostral to the hindbrain, demarcates the midbrain-hindbrain boundary, and is required for early specification of forebrain and midbrain (Acampora *et al.*, 1999). I find that the majority of Pax6-expressing pallial-like progenitors co-express Otx2, consistent with a forebrain progenitor identity (**Figure 2.2D**). Otx2 is also co-expressed by most Sox6-expressing progenitors (**Figure 2.2E**). While these data suggest that many Pax6- and Sox6-expressing progenitors resemble forebrain pallial progenitors, the absence of Otx2 co-expression in many progenitors indicates further heterogeneity not observed *in vivo*.

To assess whether downstream pallial molecular programs are intact in cells differentiating under these conditions, I assessed expression of Ngn2 in these ES-derived pallial-like progenitors. In the developing pallium, Pax6 and Sox6 are both upstream of Ngn2, a pro-neurogenic transcription factor that has cell cycle dependent expression in progenitors undergoing neurogenesis (Azim *et al.*, 2009; Kageyama *et al.*, 2008; Ma *et al.*, 2008;

Schuurmans *et al.*, 2004). I find that Ngn2 is highly expressed by cells with low Pax6 expression, suggesting that ES-derived pallial-like progenitors are undergoing neurogenesis with dynamic regulation of Pax6 and Ngn2 (**Figure 2.2F**).

To investigate whether pallial-like progenitors appropriately exclude markers of subpallial identity, I tested for molecular markers of these populations at *in vitro* day 14. Mash1, also called Ascl1, is a transcription factor expressed in the subpallium (both lateral and medial ganglionic eminences) and at the adjacent pallial-subpallial boundary; in concert with Dlx1/2, it is essential for the proper specification of subpallium-derived neurons (Long *et al.*, 2009). I find that Mash1 is co-expressed by approximately 10% of Nestin-expressing ES-derived progenitors (**Figure 2.2G**).

To investigate whether Mash1-expressing progenitors display other characteristics of subpallial progenitors, I assessed their co-expression with Gsh2 and/or Nkx2.1. Gsh2 is a transcription factor expressed by early progenitors of the lateral ganglionic eminence, and, to a lesser extent, the medial ganglionic eminence; Gsh2 functions upstream of Mash1 activation, and represses Pax6 transcription (Corbin *et al.*, 2003; Wang *et al.*, 2009; Pei *et al.*, 2011; Azim *et al.*, 2009; Batista-Brito *et al.*, 2009). Nkx2.1 is another subpallial control, expressed in the medial ganglionic eminence (Butt *et al.*, 2008). Gsh2 and Nkx2.1 are individually co-expressed with Mash1 in the subpallium, in distinct compartments; I hypothesized that some Mash1-expressing progenitors might co-express one or both these subpallial transcription factors. However, I find that Gsh2 and Nkx2.1 expression is absent in ES-derived progenitors (data not shown).

To determine whether this protocol is competent to generate cells with appropriate subpallial characteristics, I directed the ventralization of ES-derived neural progenitors with Shh agonism. In the presence of the Shh agonist Ag1.3, Pax6 expression is appropriately lost

(**Figure 2.2H**), while expression of Nkx2.1 and Gsh2, individually, is increased (**Figure 2.2I,J**). Mash1 expression was not affected (data not shown). These data provide a positive control for the absence of Gsh2 and Nkx2.1 expression with cyclopamine-mediated dorsal differentiation, confirming that subpallial gene expression by ES-derived progenitors is Shh-dependent, as expected *in vivo*. In contrast, Mash1 expression by a subpopulation of these cells appears to be independent of subpallial specification.

I next asked whether Mash1-expressing progenitors are instead pallial-like, given previous reports of cells with Mash1 expression in the dorsal pallium and at the pallial-subpallial boundary *in vivo* (Britz *et al.*, 2006; Ge *et al.*, 2006). While pallial progenitors expressing Pax6 (**Figure 2.2K**) or Sox6 (**Figure 2.2L**) are mostly distinct from Mash1-expressing progenitors, I find that approximately 15% of Pax6-expressing progenitors co-express Mash1 (**Figure 2.2K**). These findings suggest that many Mash1-expressing ES-derived progenitors are potentially pallial. This interpretation is consistent with the broad dorsalization induced by cyclopamine in ES cell differentiation, but again highlights a high degree of heterogeneity within ES-derived pallial-like progenitors by day 14.

A small subset of ES-derived neurons is neocortical, based on multiple markers

At 21 days of differentiation, approximately 30% of cells express TuJ1, and can be considered immature neurons, although this proportion is highly variable (**Figure 2.3**). Previous reports using this protocol have indicated that a higher proportion of ES-derived neurons are generated (Gaspard *et al.*, 2008; Gaspard *et al.*, 2009), which raises specific methodological points that might explain the quantitative differences I observe. First, the ES-derived cells produced by this monolayer protocol do not remain a monolayer after greater than seven days of differentiation; at later times, I observe cell overgrowth and “clumping” of cells with

heterogeneous morphologies. I use confocal imaging to more precisely localize TuJ1 staining near areas of dense cell over-growth at day 21. Counting total nuclei within aggregates of cells has not proved reliable, and I therefore excluded neurons found within these dense aggregates. Second, I maintained strict criteria for counting TuJ1-expressing neurons: TuJ1 staining must minimally encompass a hemi-circle around the nucleus, and display a polarized, neuron-like morphology. Third, because TuJ1 expression is not entirely specific to neurons (*e.g.*, TuJ1 is expressed in fibroblasts; Vierbuchen *et al.*, 2010), I excluded non-neuronal TuJ1-expressing cells based on multiple exclusion criteria: comparatively lower intensity of TuJ1 expression, fibroblast-like morphology, or any nuclei surrounded by an exceedingly high density of neurites from adjacent neurons, which can sometimes incorrectly resemble distinct neurons. Finally, TuJ1 expression is not distributed uniformly *in vitro* across a coverslip, and all characterizations were performed on selected imaging fields containing substantial numbers of neurons.

To investigate the potentially neocortical identity of these ES-derived neurons at day 21, I performed immunostaining for multiple neuronal markers. I first assessed the expression of Tbr1, which is expressed briefly by all post-mitotic pyramidal neurons generated in the developing pallium, before its expression becomes restricted to corticothalamic projection neurons (CThPN) and callosal projection neurons (CPN) in layer VI (Englund *et al.*, 2005; Hevner *et al.*, 2001). Tbr1 is expressed in few brain areas other than neocortex, and Tbr1-expressing neurons are glutamatergic (Hevner *et al.*, 2001; Bedogni *et al.*, 2010; McKenna *et al.*, 2011). Approximately 10-20% of TuJ1-expressing neurons *in vitro* also express Tbr1 (**Figure 2.3A**). Given the low percentage of ES-derived neurons expressing Tbr1, I imaged selected fields containing relatively high concentrations of Tbr1-expressing neurons for further subtype characterization.

Figure 2.3. ES-derived, Ctip2-expressing neurons are neocortical-like. (A) Tbr1-expressing neurons co-express TuJ1. (B) Ctip2-expressing neurons co-express TuJ1. (C) Ctip2-expressing neurons co-express Tbr1. (D) Ctip2-expressing cells are distinct from GAD67-expressing cells; Er81 is co-expressed in Ctip2 neurons (filled arrowheads: Ctip2/Er81; empty arrowheads: GAD67).

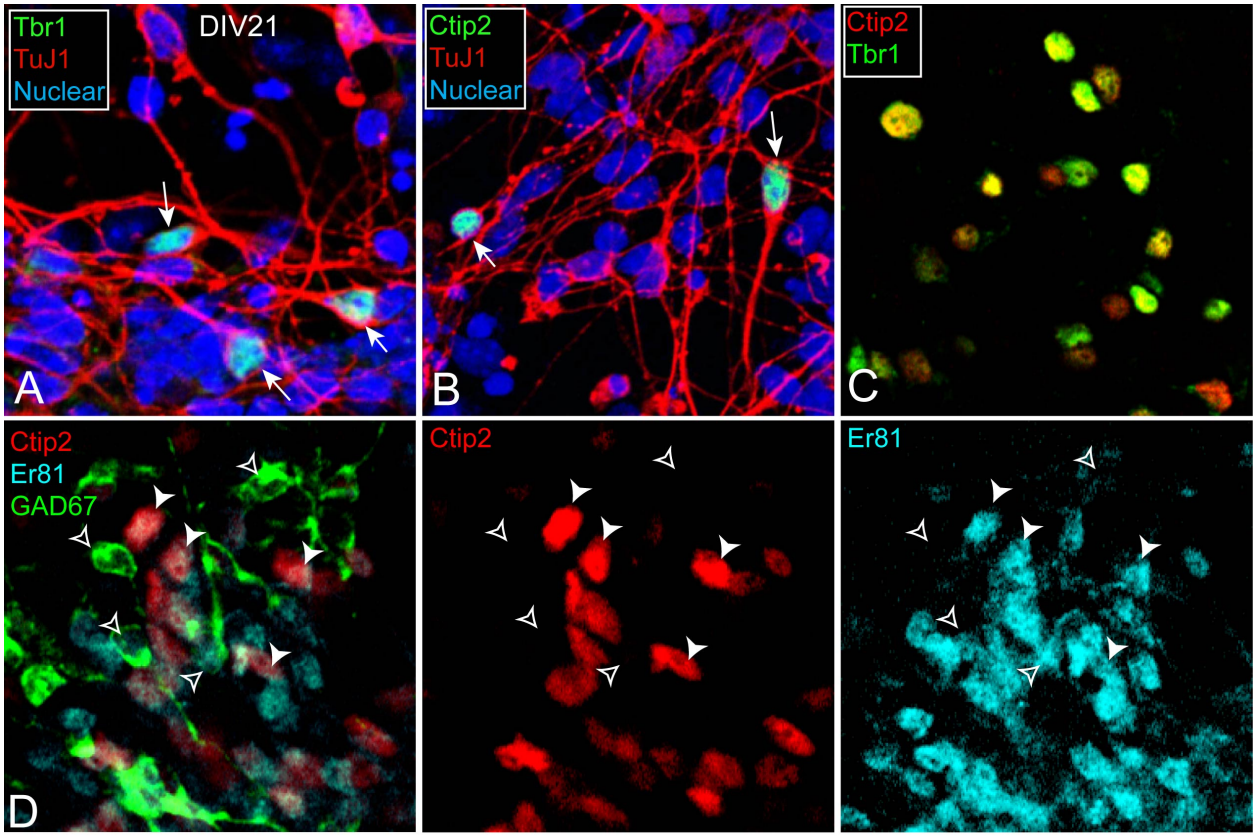

Figure 2.3 (Continued)

To identify cells with properties of early neocortical neurons, and potentially of specific deep-layer subtypes, I focused on expression of Ctip2. Like Tbr1, Ctip2 is a critical transcription factor expressed at distinct levels (off, low, high) by multiple newly post-mitotic neocortical subtypes; later in development, Ctip2 controls corticofugal projection neuron (CFuPN) axon outgrowth and fasciculation, with refined laminar expression specific to deep layers – low level by CThPN in layer VI, and high level by SCPN in layer V (Arlotta *et al.*, 2005). Expression of Ctip2 by immature CPN, and therefore co-expression with CPN marker Satb2, is lost by late embryogenesis (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Chen *et al.*, 2008). Importantly, Ctip2 is highly expressed in brain regions other than the neocortex, most highly by medium-sized spiny neurons in the striatum (Leid *et al.*, 2004; Arlotta *et al.*, 2005; Arlotta *et al.*, 2008).

I find that Ctip2, similarly to Tbr1, is expressed by a small fraction of TuJ1-expressing neurons (approximately 10-20% of neurons in selected fields containing positive Ctip2 staining) (**Figure 2.3B**). If these Ctip2-expressing neurons are neocortical-like, I hypothesized that most should also express Tbr1. Consistent with this prediction, Ctip2 and Tbr1 display nearly complete co-expression after 21 days in culture (**Figure 2.3C**). These data suggest that this sparse population of ES-derived Ctip2-expressing neurons is glutamatergic, and most closely resembles immature deep-layer projection neurons.

To rigorously test whether these Ctip2 and Tbr1 co-expressing neurons represent non-neocortical neurons, I performed co-expression analysis of Ctip2 with Er81 and GAD67. Er81 is expressed in neocortical deep layers, olfactory bulb (interneurons), amygdala, thalamus, but not in striatum (Stenman *et al.*, 2003; Yoneshima *et al.*, 2006); the intersection of Er81 and Ctip2 expression is fairly exclusive to neocortex. I find that Ctip2-expressing neurons co-express Er81 in the cytoplasm (**Figure 2.3D**), which indicates that they are not striatal. Many

important striatal genes, such as Darpp32, Foxp1, and Foxp2, are expressed both in cortex and in striatum, and we therefore examined expression of GAD67, which is expressed only by GABAergic inhibitory populations, such as medium-sized spiny neurons and subpallium-derived cortical interneurons (reviewed in Gord and Bernardo, 2011). I find that the ES-derived neurons expressing Ctip2 do not co-express GAD67, and, therefore, are not GABAergic (**Figure 2.3D**). Together, the co-expression of Ctip2, Er81, and Tbr1, and the absence of GAD67, strongly support the interpretation that a small proportion of ES-derived neurons under these relatively undirected conditions adopt properties of immature neocortical neurons *in vitro*.

Neocortical neurons are relatively immature

To investigate whether neocortical-like Ctip2-expressing neurons display appropriate features of stage-specific differentiation, I first assessed basic markers of neuronal maturation. Nearly all CNS neurons activate common programs of neuronal maturation, as marked by TuJ1, Map2, and NeuN/Fox-3 (Kim *et al.*, 2009). Very few mature neurons in the CNS lack NeuN expression, most notably Purkinje neurons and gamma spinal motor neurons (Friese *et al.*, 2009).

I find that these ES-derived neurons are relatively immature, based on the low abundance of NeuN expression after 21 or 28 days (approximately 5-10% of TuJ1-positive neurons co-express NeuN, assessed in selected fields *in vitro*). Given the importance of neuronal maturation for the timing of post-mitotic neocortical subtype refinement, I asked whether the small population of neocortical-like neurons that co-express Ctip2, Tbr1, and Er81 is mature or immature. I find that all Ctip2-expressing neurons co-express TuJ1 (**Figure 2.3B**). Approximately one third of these neurons express both Map2 and NeuN (**Figure 2.4A**). These

neurons are not uniformly or completely mature, but some display crucial hallmarks of at least early maturation.

I next investigated whether the extent of NeuN expression might indicate an equivalent stage in development. *In vivo* at E16.5-E18.5, NeuN is expressed by approximately one-third of of Ctip2-expressing neocortical neurons (**Figure 2.4B,C**). Later, at P6, all Ctip2-expressing neocortical neurons also express NeuN (**Figure 2.4D**). Between E16.5 and P6, Ctip2-expressing cortical neurons *in vivo* extend axons to their targets in the midbrain, brainstem, and spinal cord, and begin the process of pruning collateral connections (Arlotta *et al.*, 2005; Stanfield, 1992). In contrast, ES-derived neocortical neurons in culture develop to a relatively immature state most highly resembling mid-corticogenesis.

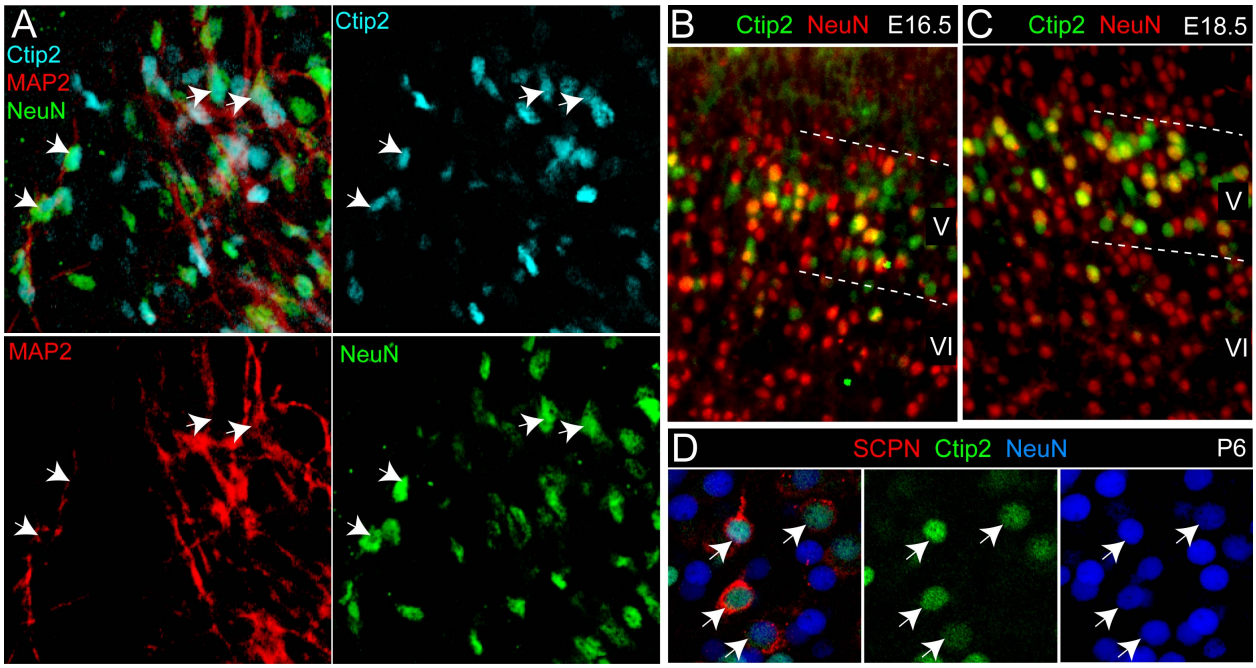

Figure 2.4. ES-derived, Ctip2-expressing neurons are immature, consistent with NeuN expression at mid-corticogenesis *in vivo*. (A) Approximately one third of ES-derived, Ctip2-expressing neurons co-express Map2 and NeuN *in vitro* (arrows: Ctip2/Map2/NeuN) (B,C) *In vivo,* NeuN is normally expressed in one third of (B) E16.5 and (C) E18.5 Ctip2-high neurons. (D) By P6, *in vivo*, all retrograde-labeled SCPN co-express Ctip2 and NeuN, indicating completion of a next stage of progressive maturation.

Impaired subtype distinction of immature ES-derived CFuPN

I next assessed whether ES-derived, Ctip2-expressing, immature neurons are appropriately molecularly distinct from other subtypes. During mid-corticogenesis, *in vivo*, when only a small percentage of neurons express NeuN, neocortical projection neurons co-express markers characteristic of multiple subtypes. By the first week of postnatal neocortical development, this molecular co-expression resolves into a refined, subtype-specific molecular identity, termed "subtype refinement" (Lai *et al.*, 2008; Joshi *et al.*, 2008; Azim *et al.*, 2009; Cederquist *et al.*, 2013; Lickiss *et al.*, 2012). One example transcription factor, Satb2, is transiently expressed by early-stage CFuPN, but is later restricted to specific expression by CPN and other associative neocortical neurons (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Lickiss *et al.*, 2012). To specifically investigate this subtype refinement of Ctip2 and Satb2 *in vivo*, for comparison to the events in culture, I assessed E16.5 neocortex and find significant Ctip2/Satb2 co-expression in layer V; these immature Ctip2/Satb2 co-expressing post-mitotic neurons consistently do not express NeuN (**Figure 2.5A**).

As a further, direct comparison, I next assessed post-mitotic subtype refinement by primary developing neocortical neurons *in vitro* using dissociated E12.5 neocortical cells cultured under the same conditions as day 14-21 ES-derived neocortical neurons. I find that these primary neurons reduce their initially high levels of Ctip2 and Satb2 co-expression, and increase the intensity of either Ctip2 or Satb2 over the course of four days *in vitro* (**Figure 2.5B-D**), confirming that primary neurons are capable of subtype-specific transcription factor refinement during maturation *in vitro*.

Figure 2.5. ES-derived, Ctip2-expressing neurons do not resolve immature projection neuron marker expression over one week *in vitro*. (A) At E16.5 *in vivo*, Ctip2 and Satb2 co-expressing neurons are relatively immature neurons, indicated by the absence of NeuN co-labeling. (B, C, D) Dissociated primary E15.5 neocortical cells initially co-express Ctip2 and Satb2, but this immature expression resolves during the course of four days *in vitro* (DIV). (E, F) Under the same culture conditions, ES-derived neocortical-like neurons co-express Ctip2 and Satb2 at 21 days (E) and this co-expression persists at 28 days (F). (G) ES-derived neocortical-like neurons also co-express Ctip2 and Ctip1 at 28 days.

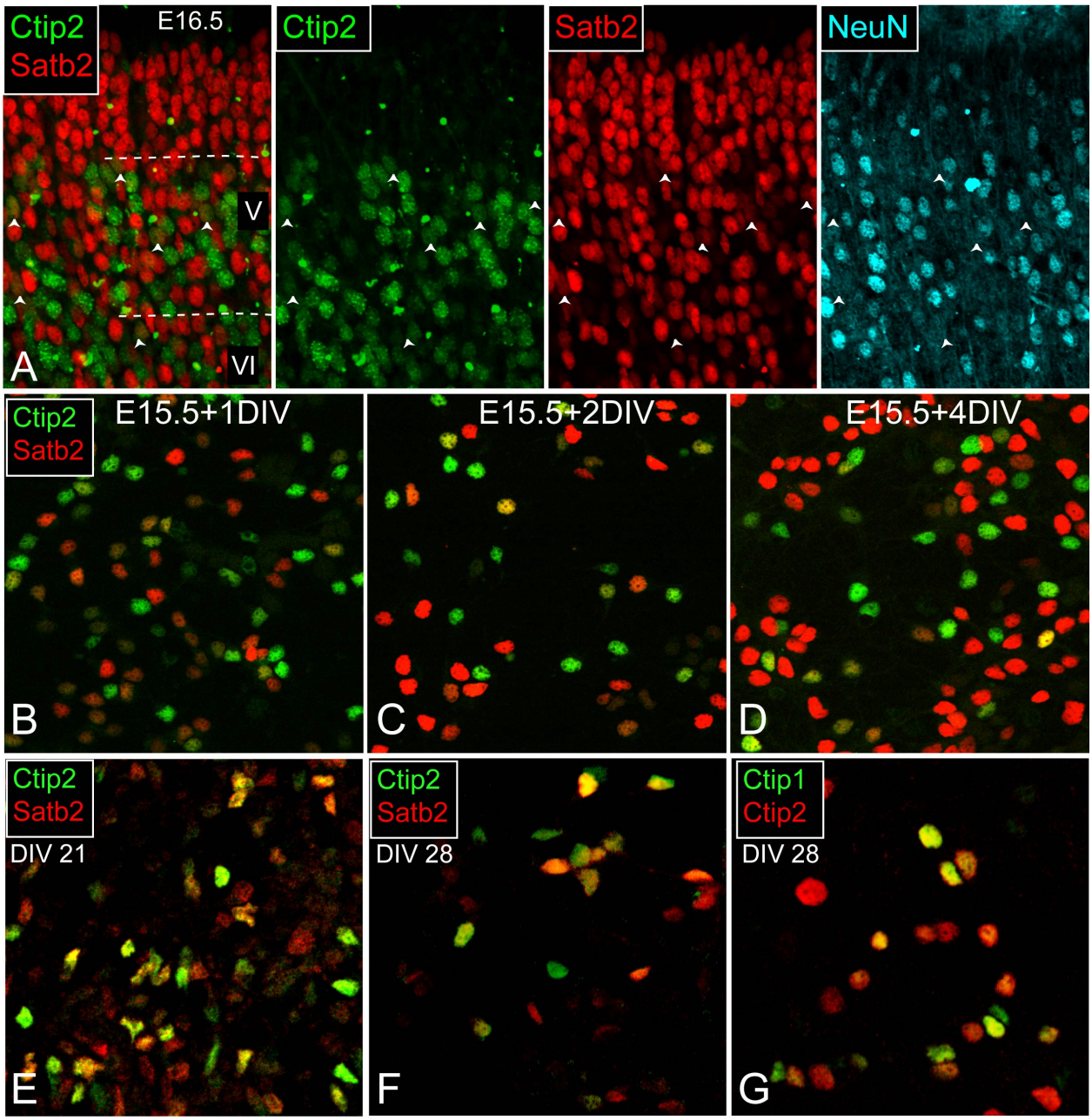

Figure 2.5 (Continued)

I then asked whether the small population of ES-derived, Ctip2-expressing, immature neocortical neurons similarly display molecular profiles consistent with mid-corticogenesis, and whether this molecular identity refines to subtype specificity over time. I find that most Ctip2-expressing neurons continue to co-express Satb2 at 21 days (one week after the onset of *in vitro* neurogenesis) (**Figure 2.5E**). Strikingly, Ctip2/Satb2 co-expression is still maintained by 28 days of post-mitotic differentiation (**Figure 2.5F**), in contrast to primary dissociated E15.5 neocortical neurons cultured for only four days under the same conditions *in vitro* (**Figure 2.4B-D**). Moreover, these ES-derived neocortical-like neurons express a continuum of low, medium, and high expression levels of Ctip2 and Satb2, in contrast to primary dissociated E15.5 neocortical neurons, which have distinctly high, low, or absent expression levels of Ctip2 or Satb2, when cultured under the same conditions *in vitro*.

To exclude the possibility of an isolated Ctip2/Satb2-specific molecular deficit, I asked whether the refinement of another subtype-specific transcription factor pair is also impaired. Ctip1 is a transcription factor that regulates both subtype- and area-specific identity; it is initially co-expressed with Ctip2 but is excluded from SCPN later in development (Woodworth *et al.*, unpublished data, 2013). Importantly, I find that Ctip1 is co-expressed by most Ctip2-expressing neurons at day 28 (**Figure 2.5G**), consistent with a broader impairment of subtype-specific molecular refinement. These data suggest that the small population of ES-derived neocortical neurons, with overlapping expression of multiple post-mitotic neocortical markers (Ctip2, Tbr1, Er81, Satb2, and Ctip1), recapitulates a stage resembling mid-corticogenesis.

Incomplete molecular area refinement of ES-derived CFuPN

It has been previously reported that some ES cell-derived neurons, when grafted in the white matter tracts ventral to the neocortex of P0/P1 mice, project axons to intra-cortical and

subcerebral (mostly visual) targets after one month (Gaspard *et al.*, 2008). The expression of a single caudal neocortex marker, CoupTF1, was used to explain these biased projection patterns. Since the time of that publication, multiple transcription factors (*e.g.*, Bhlhb5, CoupTF1, Lmo4, and Ctip1) have been characterized as important post-mitotic controls over neocortical area specification *in vivo* (Joshi *et al.*, 2008; Armentano *et al.*, 2007; Tomassy *et al.*, 2010; Huang *et al.*, 2009; Cederquist *et al.*, 2013; Woodworth *et al.*, unpublished data, 2013; Custo Greig *et al.*, unpublished data, 2013). In striking parallel to initially broad expression of genes that refine over time to define precise subtype identity, these post-mitotic area controls are initially co-expressed broadly in all neocortical areas, then become refined in expression during the first postnatal week (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). To investigate whether immature ES-derived CFuPN might have area-specific molecular identity, I assessed putative CFuPN marked by high Ctip2 expression. I find that nearly all ES cell-derived Ctip2-expressing neurons co-express CoupTF1 (**Figure 2.6A**), which is consistent with *in vivo* broad expression (caudal-high to rostral-low gradient) in the neocortex at mid-corticogenesis. In addition, Ctip2-expressing neurons also co-express Ctip1 (**Figure 2.6G**). In striking contrast, Ctip2-expressing neurons do not co-express Bhlhb5 (**Figure 2.6B**), although Bhlhb5 is expressed by other ES-derived neurons (**Figure 2.6C**). These data indicate that the absence of Bhlhb5 co-expression is inappropriate for the same stage of development *in vivo* and might represent deficits in area-specific differentiation by ES-derived neocortical neurons.

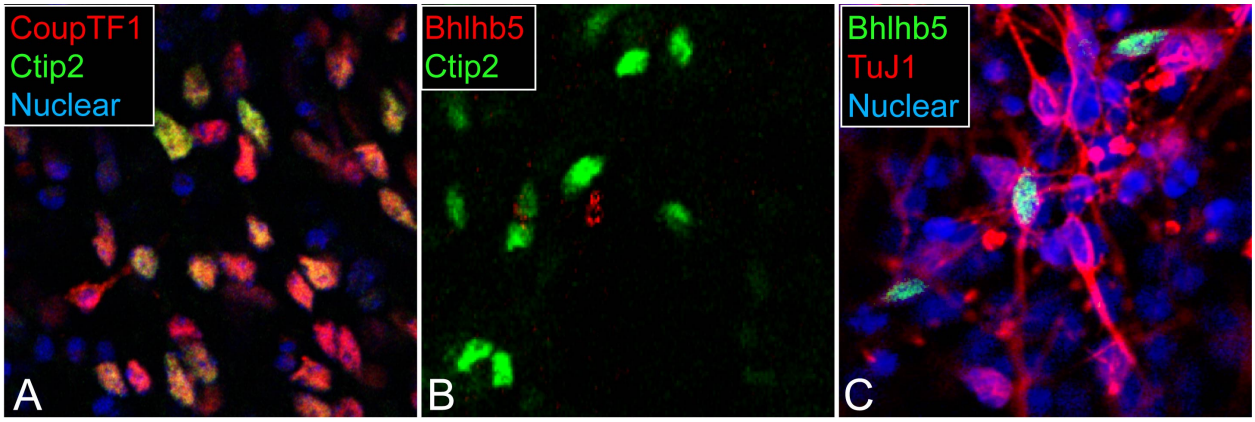

Figure 2.6. ES-derived, Ctip2-expressing neurons do not complete post-mitotic area refinements. (A) All ES-derived Ctip2-expressing neurons co-express CoupTF1. (B) All ES-derived Ctip2-expressing neurons cells exclude Bhlhb5. (C) Bhlhb5 is expressed by other ES-derived neurons (N=4; approximately 1,000 neurons were screened).

2.4 Discussion

The experiments presented here are the first to deeply investigate the differentiation of neocortical-like neurons derived from ES cells, using the current and rapidly advancing knowledge in the field, and the results identify maturation deficits of these neurons. I demonstrate the utility of coordinating markers of neuronal maturation with markers of neocortical subtypes to assess the stage and extent of neocortical differentiation. Previous reports of ES-derived neocortical neuronal subtypes have assessed the presence of individual markers or, less commonly, combinations of very limited and relatively broad markers to identify neocortical subtypes (Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Nasu *et al.*, 2012; Ideguchi *et al.*, 2010; Mariani *et al.*, 2012; Shi *et al.*, 2012; Espuny-Camacho *et al.*, 2013). However, most neocortical subtype-specific markers are only truly specific during transient developmental stages, in defined anatomical locations, and are not individually specific to the neocortex (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013).

Developmental stage-specific characterizations of ES-derived neocortical-like neurons *in vitro* suggest that these neurons most resemble *in vivo* neocortical neurons at mid-corticogenesis. This conclusion is based on three distinct developmental criteria. First, less than one third of TuJ1-expressing neocortical-like neurons express mature neuronal markers (MAP2, NeuN), consistent with the proportion of neocortical neurons that express NeuN *in vivo* at approximately E16.5-E18.5 (**Figure 2.4**). These data provide a metric for comparison to a similar developmental stage *in vivo*; I use this information to interpret the stage-specific expression of subtype markers. Second, neocortical-like neurons co-express multiple subtype-specific transcription factors (*e.g.*, Tbr1, Ctip2, Satb2, Ctip1) in a continuum of low, medium, and high expression levels consistent with *in vivo* co-expression of these genes during early- to

mid-corticogenesis, but which is in striking contrast to the more mature expression of these transcription factors by primary, dissociated E15.5 neocortical neurons cultured under the same conditions *in vitro* (**Figure 2.3; Figure 2.5**). Third, neocortical-like neurons appropriately co-express some, but not all, post-mitotic controls over area-specific differentiation (*e.g.*, CoupTF1, Bhlhb5, Ctip1; **Figure 2.6**); while this expression profile is most consistent with caudal fates, it does not reflect the broad patterns of area-specific markers during mid-corticogenesis.

Neocortical projection neurons are not the only population that displays increasingly restricted expression of subtype-specific transcription factors during maturation; indeed, spinal motor neurons (SMN) follow a similar process of refinement and diversity generation *in vivo* (Jessell, 2000; Dasen and Jessell, 2009; Alaynick *et al.*, 2011). Initially, early post-mitotic SMN express the transcription factors Hb9, Islet1, and Lhx3 (Sharma et. al., 1998), and with continued maturation and position-dependent differentiation (Sürmeli *et al.*, 2011), expression of each transcription factor becomes progressively restricted to distinct SMN subtype identities, including medial, lateral, and hypaxial motor column subtypes. However, *in vitro* subtype-specific molecular refinements by heterogeneous ES-derived SMN are not distinct at early, immature stages of differentiation (Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; Peljto and Wichterle, 2011). My findings, though directed toward characterizing neocortical neuronal identities, also reveal unresolved, immature subtype refinement *in vitro*.

Though ES-derived neocortical-like neurons recapitulate some aspects of immature neocortical development specific to a stage approximating mid-corticogenesis, these data also indicate that these neurons are “stalled” in maturation *in vitro*. This conclusion is based on the comparison of subtype refinement by primary dissociated neocortical cells and ES-derived neocortical neurons under the same culture conditions (**Figure 2.5**). The immature subtype

marker profiles in ES-derived neurons do not resolve over the course of two weeks *in vitro*, in contrast to the timing observed *in vivo*, or to primary neurons cultured with the same conditions *in vitro*. The conclusion that ES-derived neocortical-like neurons are stalled in differentiation, rather than permanently mis-specified, is supported by evidence of continued neuronal maturation, based on the extension of long-range axons to forebrain and midbrain targets but not by resolution of subtype-specific molecular markers, following transplantation into early post-natal mice (Gaspard *et al.*, 2008).

Increasingly, more refined analyses of ES-derived neuron physiology and subtype identity indicates stalled or incomplete neuronal differentiation following directed differentiation *in vitro*. For example, in one protocol of SMN generation from mouse ES cells, *in vitro* maturation is limited; only after five days of myotube co-culture do ES-derived SMN express more mature physiologic properties of post-natal spinal motor neurons (Miles *et al.*, 2004). Recently, more detailed analyses of ES-derived photoreceptor neurons (Eiraku and Sasai), midbrain-like dopaminergic neurons (Kriks *et al.*, 2011), and spinal nociceptor neurons (Chambers *et al.*, 2012), similarly suggest variability and limitations in the extent of neuronal subtype maturation *in vitro*, and following grafting *in vivo*.

I speculate that the maturation deficits in ES-derived neocortical neurons are the result of both intrinsic and extrinsic deficits. First, recent mouse studies demonstrate that the absence of specific intrinsic factors might accelerate, delay, or interrupt mature laminar or area positioning (*e.g.*, Sox5 in Lai *et al.*, 2008; FoxG1 in Miyoshi and Fishell, 2012; Bhlhb5 in Joshi *et al.*, 2008; CoupTF1 in Tomassy *et al.*, 2010, and Alfano *et al.*, 2011; Ctip1 in Woodworth *et al.*, unpublished data, 2013). The finding that Bhlhb5 is absent in ES-derived neocortical-like neurons at mid-corticogenesis is consistent with at least one intrinsic deficit in area-specific transcriptional refinement. Second, simplified growth and media conditions *in vitro* might

exclude extrinsic factors necessary for neocortical subtype distinction (reviewed in Tiberi *et al.*, 2012). Co-culturing with astrocytes might be beneficial, particularly for synaptic maturation and other refinements that occur later in postnatal development (Johnson *et al.*, 2007; Foo *et al.*, 2011), although the deficits of subtype-specific molecular refinement by ES-derived neocortical neurons occur prior to the stage that coincides with post-natal gliogenesis. Third, the absence of cell-cell interactions in adherent cell culture might impede subtype-specific refinements; strikingly, subtype marker overlap does not appear to be as severe in aggregate-based protocols of ES-derived neocortical differentiation, possibly indicating the utility of cell-cell interactions within self-organized ES-derived aggregates (Eiraku *et al.*, 2008; Nasu *et al.*, 2012). Similarly, subtype-specific maturation of ES-derived neocortical-like neurons might occur when transplanted as individually isolated neurons *in vivo,* into embryonic or post-natal neocortex, although such subtype characterizations have not been performed *in situ* (Gaspard *et al.*, 2008). Finally, intrinsic deficits in the chromatin landscape might contribute to the stalled maturation of ES-derived neocortical-like neurons; recent studies suggest that chromatin remodeling is important at multiple stages of corticogenesis (MacDonald and Roskams, 2009; Tiberi *et al.*, 2012; Baranek *et al.*, 2012). I speculate that some of these deficits might contribute to the insufficiency of ES-derived progenitors, by multiple protocols, to generate distinct superficial-layer neuron subtypes (Hansen *et al.*, 2011).

Early deficits in pallial progenitor specification might explain the sparse enrichment and stalled maturation of post-mitotic neocortical neurons. My data presented here describe heterogeneity of pallial and forebrain markers (*e.g.*, Pax6, Sox6, Otx2, and Mash1) and absence of subpallial markers in ES-derived progenitors (**Figure 2.2**). While these data suggest that dorsalization of ES-derived progenitors is highly efficient, the heterogeneity and minimally overlapping expression of multiple pallial markers (*e.g.* Pax6 and Sox6) strongly indicate an

incomplete extent of pallial differentiation by most ES-derived progenitors. In particular, the strikingly low efficiency of neocortical-like neuron generation (at most 20% of ES-derived neurons express Tbr1, Ctip2, Satb2, or Ctip1) supports the interpretation that most ES-derived pallial-like progenitors are incompletely specified. I speculate that the small population of Pax6 and Sox6 co-expressing progenitors (~20% of total progenitors; **Figure 2.2C**) most closely resembles true pallial progenitors and likely accounts for the small population of neocortical-like neurons; the prospective isolation of these ES-derived pallial-like progenitors might enable further study of neocortical subtype specification in future studies. Together, these data suggest that deficits in neocortical-like neuron subtype specification might originate with incomplete pallial progenitor specification.

Judging from the typically exceptional specificity of neocortical neuronal subtype involvement with specific neurodegenerative diseases (*e.g.*, CSMN and spinal motor neurons in ALS; cortico-striatal projection neurons in Huntington's disease), the utility of directed differentiation for studying neocortical biology, pathologic mechanisms, and potential therapies hinges on its close approximation to *in vivo* development. While the results presented in this chapter suggest caution in utilizing ES-derived neocortical cells as a model for cortical development, with further refinements these protocols might be substantially improved. For example, the same protocol of ES-derived neocortical directed differentiation was recently used as a model system to identify Bcl6 as a regulator of neocortical progenitors, and this pathway was verified *in vivo* (Tiberi *et al.*, 2012). Absent a mechanistic understanding of the deficits of ES-derived neocortical neuron differentiation, these data indicate specific directions for the continued refinement of directed differentiation to more closely approximate neocortical development. For example, deficits in the transcriptional state or chromatin landscape of ES-

derived neurons might be targeted for manipulation to enhance neocortical differentiation (Juliandi *et al.*, 2012; Chapter 3).

Taken together, the data from these experiments and from prior work by other groups indicate that ES-derived neocortical differentiation is limited *in vitro*, with multiple maturation deficits not consistent with *in vivo* development. The stage-specific, multiple-marker methodology presented here promises to be increasingly useful for the characterization of neocortical subtypes and for potentially directing the differentiation of refined subtypes. These results provide both foundation and motivation for refining, enhancing, and enriching for directed differentiation of clinically important CFuPN as a class and of distinct CFuPN subtypes.

2.5 Experimental Procedures

Cell culture and differentiation

Murine embryonic stem cells: Nagy ES cell line G4 (MMRRC stock# 011987-MU) or feeder-free E14Tg2a (Baygenomics) mouse embryonic stem cells were propagated using standard procedures (Ying *et al.*, 2003) on gelatin-coated (0.1% gelatin, StemCell Technologies) cell culture treated plastic dishes. Nagy ES cells were cultured on mouse embryonic fibroblast feeder cells (Millipore EmbryoMax PMEF-N). Mouse embryonic stem cell media is GMEM (Invitrogen) supplemented with 10% ESC-certified fetal bovine serum (vol/vol, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM β-mercaptoethanol (Sigma), 50 U/mL penicillin/streptomycin and 1000 U/mL leukemia inhibitor factor (ESGRO).

For differentiation, Nagy G4 and E14Tg2a ES cells were plated at low density (5,000 cells/cm^2) on gelatin-coated plastic dishes in ES cell medium, and cultured as described (Gaspard *et al.*, 2009). Briefly, ESCs were trypsinized, dissociated, and plated on gelatin-coated cell culture plates. Medium was changed to DDM after one day. DDM consists of DMEM/F12 (Invitrogen-Gibco) supplemented with N2 supplement (N2 supplement consists of 8.61uM insulin, 1mM transferrin, 2uM progesterone, 10mM putrescine and 3uM selenite; Invitrogen-Gibco), 2mM glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 0.5 mg/ml bovine serum albumin fraction V (all from Invitrogen-Gibco), and 0.1mM β-mercaptoethanol (Sigma).

Cyclopamine (Calbiochem) or Ag1.3 (gift from Lee Rubin, Harvard University) was added from day 2 to day 10 in the differentiation medium at a final concentration of 1uM. After 10 to 14 days of differentiation, cells were trypsinized, dissociated and plated on poly-

lysine/laminin (Becton-Dickinson) coated glass coverslips and allowed to grow for 4–14 days in N2B27 medium. N2B27 medium consists of a 1:1 mixture of DDM and Neurobasal that is supplemented with B27 (without vitamin A; Invitrogen-Gibco) and 2 mM glutamine.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (wt/vol) for 30 min, and washed three times in phosphate-buffered saline (PBS). For primary antibodies, I used rabbit antibody to Pax6 (Covance), rabbit antibody to Sox6 (1:500, Abcam), mouse antibody to Mash1 (1:500, BD), rabbit antibody to Gsh2 (1:500, Abcam), mouse antibody to Nkx2.1/TTF1 (1:5,000 BioPat), rabbit antibody to Tbr1 (1:500, Abcam), rat antibody to Ctip2 (1:500, Abcam), mouse antibody to Satb2 (1:200, Abcam), rabbit antibody to Er81 (1:100, Abcam), mouse antibody to GAD67 (1:1000, Chemicon), rabbit antibody to TuJ (1:1000, Sigma), mouse antibody to TuJ (1:1000, Covance), mouse or rabbit antibody to Map2 (1:500, Sigma), mouse antibody to NeuN (Chemicon or Millipore), mouse antibody to Ctip1 (1:250, Abcam), goat antibody to Bhlhb5 (1:300, Santa Cruz), rabbit antibody to CoupTF1 (1:500, gift of M. Studer lab). Secondary antibodies were from the Invitrogen Molecular Probes Alexa series. Nuclei were stained with Hoechst#33342 (1:3,000, Sigma).

Wide-field image acquisition was performed using a Nikon 90i epifluorescence microscope with a Clara DR-328G cooled CCD digital camera (Andor Technology, Belfast, Northern Ireland). Confocal imaging was performed with a BioRad Radiance 2100 Rainbow laser-scanning confocal microscope based on a Nikon E800 microscope. Images were assembled in Adobe Photoshop and Illustrator (CS3, CS5), with adjustments for contrast, brightness, and color balance to obtain optimal visual reproduction of data.

Mice

All mouse studies were approved by the Massachusetts General Hospital and/or Harvard University IACUCs, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Wild-type CD1 mice were used in all experiments (Charles River Laboratories).

Brains were fixed using standard methods (Fricker-Gates *et al.*, 2002; Arlotta *et al.*, 2005). Briefly, brains were fixed by trans-cardial perfusion with PBS– heparin (10 U/ml), followed by 4% paraformaldehyde, and post-fixed overnight at 4°C in 4% paraformaldehyde. Brains were sectioned coronally at 50 um on a vibrating microtome (Leica). Coverslips or floating sections were blocked in 1% BSA (Sigma) and 0.1% Triton X-100 (Sigma) for 20min at room temperature, before incubation in primary antibody.

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Chapter 3

Sirt1 inhibition promotes refinement of subcerebral projection neuron identity among heterogeneous neocortical-like neurons generated from mouse embryonic stem cells

Publication

This chapter is in the process of being prepared for manuscript submission in summer, 2013. Experiments are ongoing.

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Author contributions

I initiated the project independently. Wataru Ebina in Derrick Rossi's laboratory provided expertise for the application of synthetic modified mRNA technology, reagent support, and discussion for this work. Data from this chapter is not incorporated into his dissertation research. Tony Arvanites and Lance Davidow in Lee Rubin's laboratory provided expertise for small molecule screening, reagent support, and discussions.

3.1 Abstract

Corticofugal projection neurons (CFuPN) are the broad class of neocortical projection neurons that send their axons to sub-cortical targets including the thalamus, striatum, brainstem, and spinal cord. Among the many types of CFuPN, subcerebral projection neurons (SCPN) specifically send their axons to sub-cerebral targets in the brainstem and spinal cord. Importantly, the expression of a single transcription factor, Fezf2, is both required and sufficient for SCPN specification from neocortical progenitors *in vivo*. In contrast, callosal projection neurons (CPN) comprise a second broad class of projection neurons that differ from CFuPN (and thus, SCPN), in that they send inter-hemispheric axons within the neocortex and do not express Fezf2.

During late embryonic development of the neocortex, these distinct broad classes of projection neurons initially express overlapping molecular controls that later refine to specific classes and subtypes as increasingly diverse neurons mature. Such molecular refinements are largely absent in heterogeneous neocortical-like neurons spontaneously generated by an established protocol of embryonic stem (ES) cell differentiation (**Chapter 2**). Given probable deficiencies in chromatin remodeling during maturation of ES-derived neocortical-like neurons, I investigated whether distinct chromatin modifiers might promote SCPN-specific differentiation among heterogeneous, maturation-stalled neocortical-like neurons spontaneously generated from ES cells.

Using a combination of “high content” screening technologies, and recently developed synthetic modified mRNA (modRNA; pioneered by the Derrick Rossi laboratory at Harvard; Warren *et al.*, 2010), I investigated whether chromatin remodeling might enhance Fezf2-mediated SCPN specification among ES-derived neocortical-like neurons. I screened a library

of eighty small molecules that target known chromatin remodeling enzymes for SCPN differentiation, in the context of transient Fezf2 expression.

The data show that the inhibition of a specific histone deacetylase, Sirtuin 1 (Sirt1), enhances the refinement of SCPN identity, as assessed by positive and negative molecular markers of SCPN identity in individual cells. Using expression analysis *in vivo* during mouse cortical development, I find that this effect of Sirt1 inhibition in promoting ES-derived SCPN differentiation is consistent with specific absence of Sirt1 expression by SCPN during their late embryonic and post-natal differentiation *in vivo*. Moreover, Sirt1 inhibition by small molecules or siRNA knockdown enhances SCPN subtype refinement by primary dissociated E12.5 neocortical cells, reinforcing the biological relevance of the work with ES-derived neurons.

Together, these data provide the first report of subtype-specific expression and function of a chromatin-remodeling enzyme in the mouse neocortex, applicable to directing ES cell differentiation. Moreover, these findings indicate that histone modifications contribute to the molecular refinement of neuronal subtype identity during neocortical development.

3.2 Introduction

Corticofugal projection neurons (CFuPN) are the broad class of neocortical neurons that send axonal projections from the neocortex to distal targets in the thalamus, midbrain, hindbrain, and spinal cord (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012). A defining molecular feature of CFuPN is the early and temporally dynamic expression of Fezf2, a required transcription factor that specifies their anatomical and molecular identity (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Shim *et al.*, 2012). The expression of Fezf2 is highest in neocortical subcerebral projection neurons (SCPN), which are a subset of CFuPN with axonal projections to targets that are more caudal to the thalamus, beyond the cerebral peduncle (Molyneaux *et al.*, 2005).

Although Fezf2-expressing SCPN are among the first neurons generated in the neocortex, they undergo progressive transcriptional refinements that continue for multiple weeks prior to the acquisition of a stable molecular identity (Azim *et al.*, 2009; Cederquist *et al.*, 2013). SCPN identity is initially masked by the co-expression of multiple subtype-specific markers of inter-hemispheric callosal projection neurons (CPN), including Satb2 (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Azim *et al.*, 2009; Woodworth *et al.*, 2012; **Chapter 2**). Following continued maturation, Fezf2-expressing SCPN stably repress the expression of Satb2 and other markers of CPN identity. SCPN further resolve into distinct subtypes of projection neurons with laminar- and area-specific properties (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). While multiple molecular controls over the post-mitotic regulation of neocortical subtype refinement have been identified (Joshi *et al.*, 2008; Lai *et al.*, 2008; Tomassy *et al.*, 2010; Miyoshi and Fishell, 2012; Cederquist *et al.*, 2013), it is currently unclear how these subtype-specific post-mitotic refinements are regulated.

Multiple epigenetic mechanisms might help generate diverse neocortical subtypes and enable their refinement and maturation (Kishi and Macklis, 2004; MacDonald and Roskams, 2008, 2009; Kishi *et al.*, 2012; Yip *et al.*, 2012). Importantly, neocortical subtype-specific mechanisms have been recently identified and are being investigated. First, Satb2, a required transcriptional control over callosal projection neuron (CPN) development, is a CPN-specific matrix-attachment region (MAR) binding protein with punctate intra-nuclear localization consistent with specific chromatin binding (Britanova *et al.*, 2005; Gyorgy *et al.*, 2008; Alcamo *et al.*, 2008; Britanova *et al.*, 2008); MAR binding proteins, such as Satb2, can mediate long-range interactions of enhancer sites with promoters (Yasui *et al.*, 2002; Cai *et al.*, 2003; Dobreva *et al.*, 2003). Second, Ski, a subtype non-specific transcriptional cofactor, binds Satb2 in CPN and recruits the nucleosome remodeling and histone deacetylase (NuRD) complex (Baranek *et al.*, 2012). Additionally, studies in non-neocortical cells have suggested epigenetic mechanisms for transcriptional regulators with roles in neocortical subtype-specific differentiation. The transcription factors Ctip2 and Ctip1 are differentially expressed in the neocortex, with specific roles in directing the precision of SCPN and CPN differentiation (Leid *et al.*, 2004; Arlotta *et al.*, 2005; Woodworth *et al.*, unpublished data, 2013). These transcription factors have been demonstrated to interact with the NuRD complex (Topark-Ngarm *et al.*, 2006; Cismasiu *et al.*, 2005) and Sirt1 (Senawong *et al.*, 2003; Senawong *et al.*, 2005) to mediate chromatin remodeling in non-neocortical cells. Together, these reports suggest that chromatin remodeling might contribute to the post-mitotic refinement of neocortical projection neuron subtypes, particularly in the refinement of Ctip2-expressing SCPN from Satb2-expressing CPN.

Embryonic stem (ES) cell-based models of neocortical differentiation are emerging as a useful tool to study the roles of chromatin modifications in neocortical development. For

example, in a recent genetic screen of ES-derived neocortical cells, Bcl6 was identified as a pro-neurogenic transcription factor that recruits the histone deacetylase Sirtuin1 within neocortical progenitors (Tiberi *et al.*, 2012). In a related protocol of neocortical directed differentiation from ES cells, a broad histone deacetylase inhibitor, valproic acid, was shown to increase the population of neurons expressing Cux1, which, as a single marker *in vitro*, is potentially indicative of CPN identity (Juliandi *et al.*, 2012). These studies suggest that ES-derived neocortical cells can potentially identify epigenetic mechanisms of post-mitotic differentiation to bias differentiation toward specific post-mitotic fates.

To specifically study SCPN post-mitotic refinement, I first hypothesized that the appropriately timed induction of Fezf2 in ES-derived neocortical progenitors might bias these cells toward SCPN fate. Fezf2 mis-expression by non-SCPN forebrain cell types (*e.g.*, superficial layer neocortical progenitors, callosal projection neurons, or striatal medium spiny neurons) induces SCPN molecular identity within these cells and redirects their axonal projections to targets in the midbrain and hindbrain (Molyneaux *et al.*, 2005; Chen *et al.*, 2008; Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013).

Although Fezf2 is a versatile transcriptional regulator that can act in numerous forebrain subtypes, its potency is dependent on molecular context and timing. The expression of Fezf2 is critically regulated by multiple Sox family transcription factors on upstream enhancer sites (Lai *et al.*, 2008; Shim *et al.*, 2012), and its transcriptional activity is likely regulated by specific cofactors in the molecular context of forebrain development. In the absence of these forebrain-specific factors, Fezf2 mis-expression at early stages of ES cell differentiation does not drive SCPN molecular identity (Wang *et al.*, 2011; Sadegh, unpublished data, 2011). By extension, incompletely specified ES-derived forebrain-like cells might not express the context-dependent factors needed for Fezf2-mediated fate specification.

While protocols for directing neocortical differentiation from ES cells have succeeded in replicating some of the molecular characteristics of neocortical development (Eiraku *et al.*, 2008; Gaspard *et al.*, 2008), the enrichment and mature refinement of neocortical subtypes is incomplete (**Chapter 2**). These data suggest that ES-derived neocortical cells are unlikely to have a sufficiently permissive molecular context for Fezf2-directed differentiation of SCPN.

I hypothesized that chromatin remodeling by incompletely specified ES-derived neocortical progenitors might promote a permissive molecular context for Fezf2 induction and directed SCPN subtype refinement. This concept of "epigenetic priming" (term coined by Scandura *et al.*, 2011) is increasingly accepted as a strategy of partially reversing an epigenetically-repressed transcriptional state, as demonstrated by the neuronal reprogramming of germ cells in *C. elegans* (Tursun *et al*., 2011; Patel *et al*., 2012) and by the promotion of induced pluripotent stem (iPS) cell reprogramming (Papp and Plath, 2013).

To identify candidate chromatin remodeling enzymes, I conducted a high-content screening experiment on ES-derived neocortical cells, using a library of small molecules that modulate known epigenetic enzymes. Importantly, I developed an assay to identify molecular refinement of distinct post-mitotic subtypes.

From this screen, I identify the histone deacetylase Sirtuin1 (Sirt1) as a repressor of Fezf2-mediated SCPN refinements. Small molecule inhibitors of Sirt1 (*e.g.*, EX-527, CHIC-35) help refine the identity of Ctip2-expressing SCPN from Ctip2/Satb2 co-expressing immature neocortical neurons *in vitro*. Moreover, I verify *in vivo* Sirt1 subtype specificity in post-mitotic neocortical projection neuron subtypes during late embryonic and early post-natal development. Together, these data implicate differential chromatin remodeling as an important epigenetic mechanism of neocortical subtype refinement both *in vivo* and for *in vitro* directed differentiation.

3.3 Results

Synthetic modified mRNA induces transient and dose-dependent gene expression in ES-derived neocortical-like cells

Synthetic modified mRNA (modRNA) enables precision over gene dosage and timing in a variety of mouse and human cell types (Warren *et al.*, 2010). Because modRNA does not integrate into the genome and has a limited duration of expression (approximately 2 days), modRNA enables transient induction of genes with minimal disruption of ES-derived cells. This technology was powerfully demonstrated to reprogram fibroblasts to induced pluripotent stem (iPS) cells, and to direct the differentiation of myotubes from these iPS cells (Warren *et al.*, 2010). Other related modRNA technologies have also successfully generated dose- and time-limited expression of proteins *in vitro* and *in vivo* (Angel and Yanik, 2010; Kormann *et al.*, 2011; Petsch *et al.*, 2012; Mays *et al.*, 2013).

I investigated whether modRNA transfection results in time- and dose-sensitive protein expression by differentiating feeder-free E14Tg2a mouse embryonic stem (ES) cells in an established monolayer protocol that generates heterogeneous, maturation-limited, neocortical-like neurons (Gaspard *et al.*, 2008; Gaspard *et al.*, 2009; **Chapter 2**). In agreement with the prior literature, I find a dose-dependent intensity of GFP expression after modRNA transfection of ES-derived cells at the peak of pallial-like differentiation at day 14; modRNA-induced GFP expression peaks between 12-24 hours, with sharp reduction of expression by 48hrs (**Figure 3.1A,B**). I also find that modRNA transfection is not biased to a specific neural population; modRNA transfects Nestin-expressing neural progenitors, TuJ1-expressing neurons, and other cells (**Figure 3.2**). Overall, these data indicate that modRNA technology enables unbiased, dose- and time-dependent gene expression in ES-derived cells, including progenitors and neurons.

Figure 3.1. Synthetic modified RNA (modRNA) enables precision over gene dosage and timing in ES cell-derived populations, and is functional when expressed *in vivo*. (A) Native GFP expression is detected in 25-50% of cells after 24hrs; the intensity of expression is dose-dependent, over the range of 0, 2, 4 ug modRNA transfection. (B) GFP mod-RNA expression is time-dependent over 48hrs. (C) In the brain of a viable mouse embryo within the uterus, GFP modRNA is injected into the forebrain lateral ventricles (cross-section is shown) and is directionally electroporated (five 50ms square-wave pulses at 30 V) into the pallium (positive paddle above dark blue colored tissue). (D) Following GFP modRNA *in utero* electroporation, GFP is expressed after eight hours, and is restricted to Ki67-expressing progenitors of the pallium. (E) Sagittal section of an E17.5 Fezf2-null mouse, following E12.5 in utero electroporation of Fezf2 modRNA (0.8ug/uL) and tdTomato plasmid (1ug/uL), shows targeted tdTomato fluorescence appropriately restricted to the rostral pallium. (F) Zoom showing tdTomato expression in the cortical plate. (G) As expected in the Fezf2-null mouse, many tdTomato axons project across the cerebral commissures, including anterior commissure (depicted) and corpus callosum in (E). (H) Strikingly, some tdTomato axons project caudal to the thalamus, indicating rescue of E12.5 SCPN. (I) A smaller subset of these tdTomato axons reaches the cerebral peduncle (N = 3).

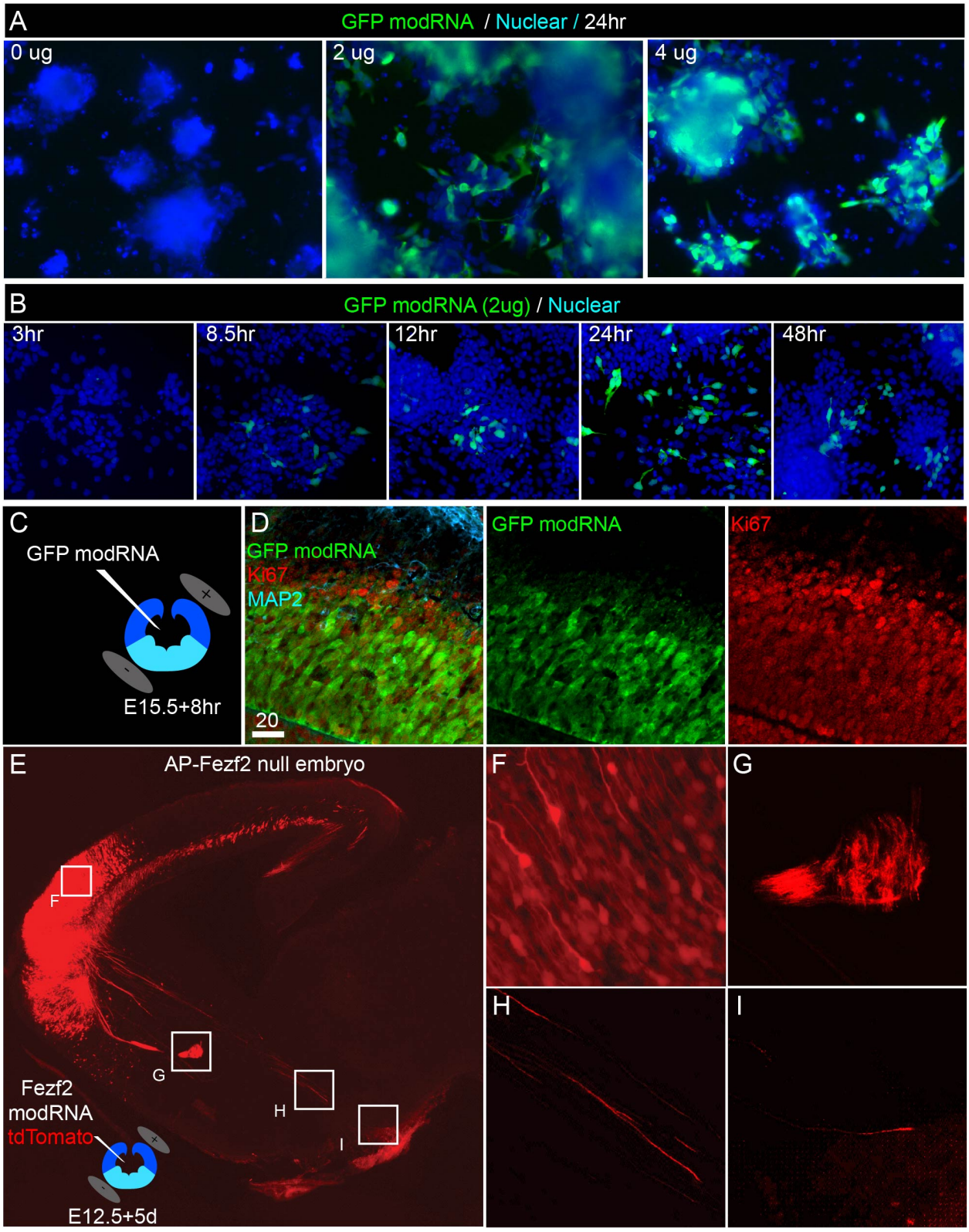


Figure 3.1 (Continued)

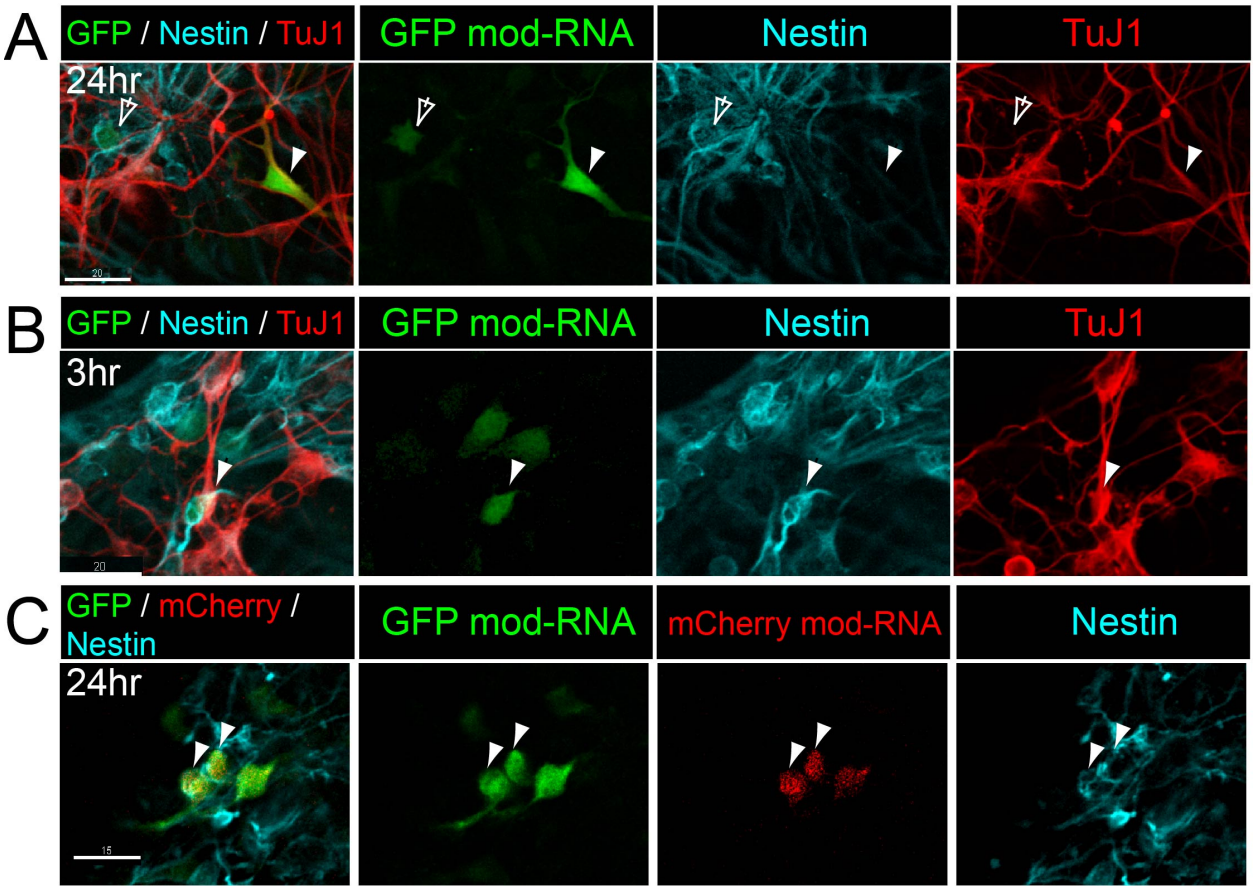


Figure 3.2: modRNA transfection is not biased to a specific neural population. (A) After 24hrs, GFP modRNA transfects progenitors (Nestin-expressing, empty arrows), neurons (TuJ1-expressing, filled arrows), and other cells. (B) GFP modRNA is expressed in Nestin-positive cells after three hours. (C) mCherry and GFP mod-RNA are co-expressed in the same cells.

Transient Fezf2 expression rescues SCPN *in vivo* within a stage-specific molecular context

Prior to investigating the expression of Fezf2 in ES-derived neurons, I first asked whether *in vivo* Fezf2 expression by modRNA is functionally comparable to established protocols of Fezf2 expression by plasmid vectors. Specifically, with a single induction at a relatively low dose, I asked at what age of development is a transient pulse of Fezf2 sufficient to drive SCPN specification *in vivo.* To address this question, I hypothesized that transient Fezf2 modRNA expression by Fezf2-null neocortical progenitors rescues Fezf2-dependent SCPN differentiation. In Fezf2-null mice, E12.5 post-mitotic neurons do not differentiate into SCPN, and instead are re-specified to CPN; Fezf2-deficient neocortical neurons do not project axons caudal to thalamus. Fezf2 expression by plasmid electroporation in Fezf2 null mice at E12.5 can rescue normal SCPN specification and their projections to the distal hindbrain (Azim, 2009, Dissertation). At E13.5, E15.5, and later ages, Fezf2 mis-expression by plasmid electroporation can redirect commissural neurons to SCPN targets in the hindbrain (Molyneaux *et al.*, 2005; Chen *et al.*, 2008; Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013).

I first assessed the extent of modRNA electroporation *in vivo*, and found that *in utero* electroporation of GFP modRNA into the pallium (**Figure 3.1C**) is limited to the mitotic (Ki67-expressing) ventricular zone (**Figure 3.1D**). Next, in Fezf2 null mice at E12.5, I found that *in utero* electroporation of Fezf2 modRNA (with tdTomato plasmid for long-term visualization of axons; **Figure 3.1E,F,G**) rescues a subset of SCPN that project beyond the thalamus to the cerebral peduncle (**Figure 3.1H,I**), comparable to plasmid-mediated Fezf2 expression after the same number of days. In contrast, *in utero* electroporation of Fezf2 modRNA does not induce

SCPN at E15.5 (data not shown), possibly due to lower modRNA dosage (0.8ug/uL) or duration of expression (approximately 48hrs) as compared to Fezf2 plasmid electroporation.

These data indicate that Fezf2 modRNA is functional within the molecular context of e12.5 neocortical progenitors. Importantly, a single transient dose of Fezf2 is sufficient to rescue Fezf2-null SCPN at E12.5; at later ages, a higher dose or duration of expression (as provided by a plasmid vector) is required for re-specifying alternate neocortical subtypes (Molyneaux *et al.*, 2005; Chen *et al.*, 2008; Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013).

Transient Fezf2 expression alone minimally refines SCPN differentiation within ES-derived neocortical-like neurons

I next asked whether Fezf2 modRNA alone can induce SCPN-specific differentiation of ES-derived neocortical cells. I hypothesized that Fezf2-directed SCPN differentiation would be limited to the sparse population of ES-derived cells with an appropriate forebrain-specific molecular context. To assess the small population of ES-derived Ctip2- and Satb2-expressing neocortical-like neurons, I used randomized, automated imaging (at 20x magnification, on approximately 40 fields per well; ~5,000 cells), to count sufficient numbers of neocortical-like neurons for these analyses. A high threshold for positive antibody staining was manually established (compared to baseline staining without primary antibody) because populations of ES-derived neurons expressed a continuum of transcription factor staining intensities, in contrast to populations of primary dissociated E15.5 neocortical neurons, which displayed typically bimodal staining (low, high). Using this methodology, I found that 48hrs after Fezf2 modRNA transfection in ES-derived neocortical cells at *in vitro* day 18, between time points of peak Nestin and TuJ1 expression, overall numbers of Ctip2- or Satb2-expressing neocortical

neurons are not increased (**Figure 3.3A**). Normally, Ctip2 expression occurs within 48hrs of Fezf2 expression (data not shown). Given the low dose and transient expression of Fezf2 modRNA, the absence of statistically significant Ctip2 enrichment by a largely heterogeneous, immature, and incompletely specified population of ES-derived neurons (**Chapter 2**) is not surprising.

I next hypothesized that transient Fezf2 modRNA expression biases subtype refinement toward SCPN among the relatively small fraction of pre-existing immature neocortical-like neurons, which are the cells most likely to have a permissive molecular context to respond to Fezf2. Specifically, I asked whether Fezf2 modRNA transfection increases Ctip2 expression at the expense of Satb2. Using the refinement of Ctip2 expression (ratio of Ctip2-only expressing cells to Ctip2/Satb2 dual expressing cells) as an indication of SCPN identity refinement, I found that Fezf2 + GFP promotes SCPN subtype refinement compared to GFP alone, though not with statistical significance (**Figure 3.3A**). Among Ctip2/Satb2 dual-expressing neocortical-like neurons transfected with Fezf2 and GFP, the average intensity of Ctip2 expression increases (though not with statistical significance), while average Satb2 expression is unchanged, compared to GFP alone (**Figure 3.3B**). These data suggest that a single, transient dose of Fezf2 is not sufficient to refine SCPN identity by ES-derived neurons with an immature neocortical-like molecular context. These data are consistent with the inability of a single Fezf2 transfection to redirect E15.5 CPN axons to subcerebral targets *in vivo* (data not shown, see also **Figure 3.1C-I**). Therefore, the absence of statistically significant Fezf2 modRNA-mediated enhancement of SCPN differentiation within heterogeneous, maturation-stalled, ES-derived, neocortical-like neurons suggests that additional, potentially complementary manipulations are needed to more completely direct SCPN differentiation.

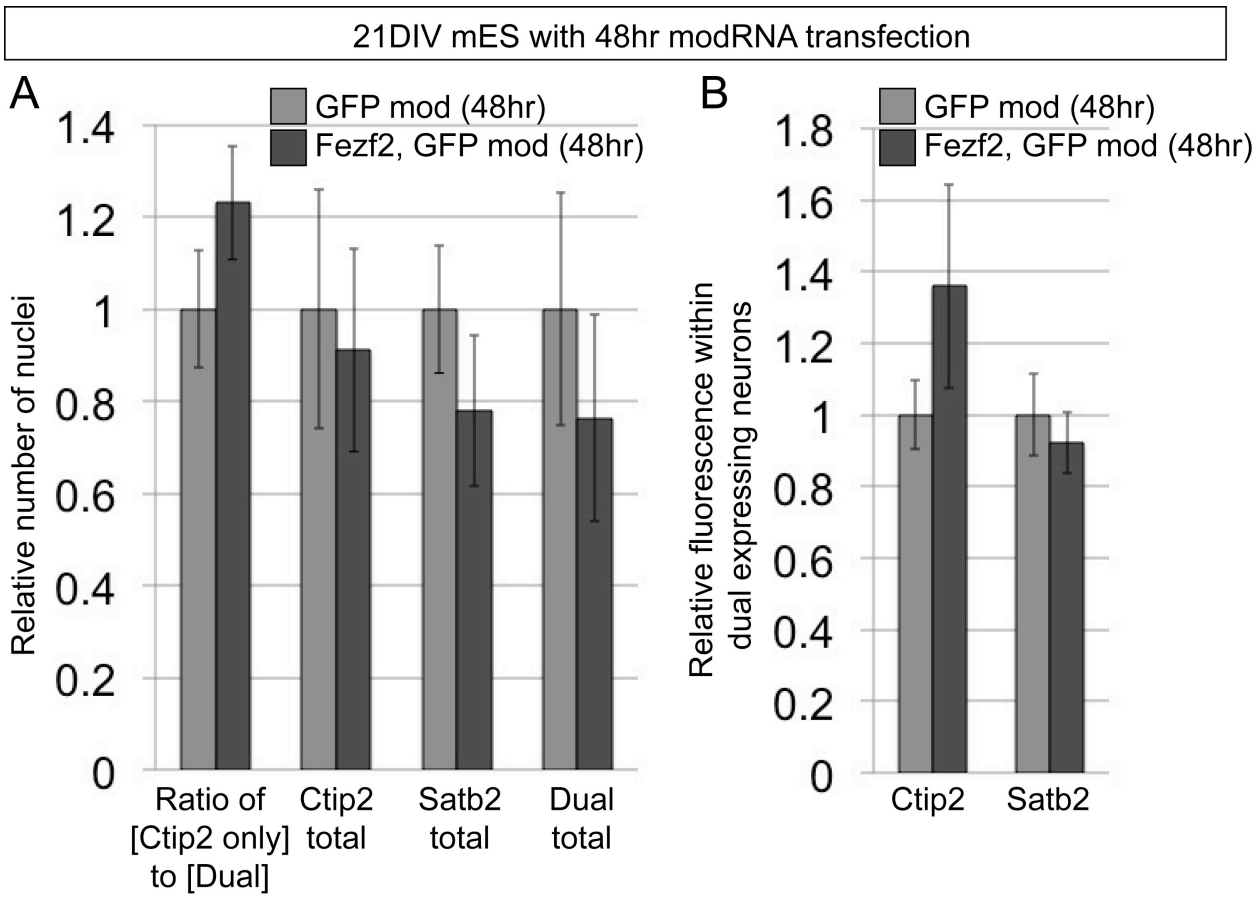


Figure 3.3. Fezf2 induction in ES-derived neurons does not significantly increase subtype distinction. (A) At 21 days in vitro (21DIV), the ratio of Ctip2 (Satb2 negative) neurons to Ctip2/Satb2 dual expressing neurons is increased by approximately 20% (though not with statistical significance) 48hrs after Fezf2 and GFP co-transfection (dark grey) relative to GFP transfection alone (light gray). Total number of Ctip2-expressing neurons is largely unaffected, whereas total Satb2-expressing and Ctip2/Satb2 dual expressing neurons decrease in number (though not with statistical significance). (B) The intensity of Ctip2 expression within Fezf2+GFP modRNA-treated neurons increases relative to GFP modRNA controls. Data are presented as mean +/- s.e.m. (N=3; approximately 5,000 cells per condition, from 40 randomly sampled fields at 20x magnification).

Small molecule screening of ES-derived neocortical-like neurons identifies Sirt1

I next asked whether remodeling the epigenetic state might enable a higher proportion of neocortical-like neurons to respond to Fezf2-induced SCPN subtype refinement. To address this question, I designed a combined small molecule screening / Fezf2 induction approach: directed differentiation to day 14 neocortical progenitors; small molecule library addition to media for four days; Fezf2 modRNA transfection, followed by fixation after 48hrs (**Figure 3.4A**). I designed a custom library of eighty small molecules modulating known epigenetic enzymes, with targets including histone deacetylases, methyltransferases, and kinases (**Figure 3.4B**). Using automated imaging and threshold analyses, I quantified the expression of Ctip2 and Satb2 within individual neurons (**Figure 3.4C,D**).

I used multiple selection criteria to identify candidates. In the first assay, I found that multiple Sirtuin modulators enhanced or inhibited Fezf2-mediated subtype refinement, as indicated by the ratio of Ctip2(+)/Satb2(-) neurons to Ctip(+)/Satb2(+) neurons (**Figure 3.5A**). Focusing on the small molecules that enhanced Fezf2-mediated refinement of Ctip2(+)/Satb2(-) expression, I then asked which small molecules increased the number of Ctip2 expressing neurons, relative to Fezf2 induction alone (**Figure 3.5B**). Lastly, selecting for compounds that showed higher numbers of Ctip2-expressing cells, I asked which compounds maintained or decreased the number of Satb2 expressing neurons dependent on Fezf2 induction alone (**Figure 3.5C**). With these multiple criteria, the Sirt1 inhibitor EX-527 emerged as a top candidate regarding induction of molecular characteristics of SCPN. As an internal control, I compared the activity of EX-527 to other sirtuin inhibitors and activators. Non-specific sirtuin inhibitors (nicotinamide, forskolin, and tenovin-6) did not increase SCPN refinement. Conversely, the Sirt1-specific activator (CAY10591) displayed antagonism to SCPN refinement.

Figure 3.4: Design of high throughput screening protocol to assess subtype refinement within neurons. (A) Schematic of screening strategy in 96-well plates. Monolayer ES cell differentiation to telencephalic progenitors is followed by the addition of a custom small molecule library; composition of this library is described in (B). After small molecule incubation for six days, each well is transfected with Fezf2 modRNA. Two days later, cells are fixed and immunostained for Ctip2, Satb2, and Ctip1. Automated imaging and fluorescence intensity thresholding algorithms distinguish and count neurons; example of this in (C).
(B) The composition of a custom set of 80 chemicals affecting histone deacetylases, methyltransferases, and kinases is depicted in this pie chart.
(C) Automated imaging and counting algorithms identify Ctip2 and Satb2 expression levels. Manually determined thresholds distinguish [Ctip2(+) / Satb2(-)] neurons (pseudo-colored green) from dual expressing [Ctip2(+) / Satb2(+)] neurons (pseudo-colored yellow).
(D) Thresholds for nucleus area and mean staining intensity were manually set using Columbus software (PerkinElmer). Examples are shown for the gating of high Ctip2 and low Satb2 expression, with screenshots from the software.

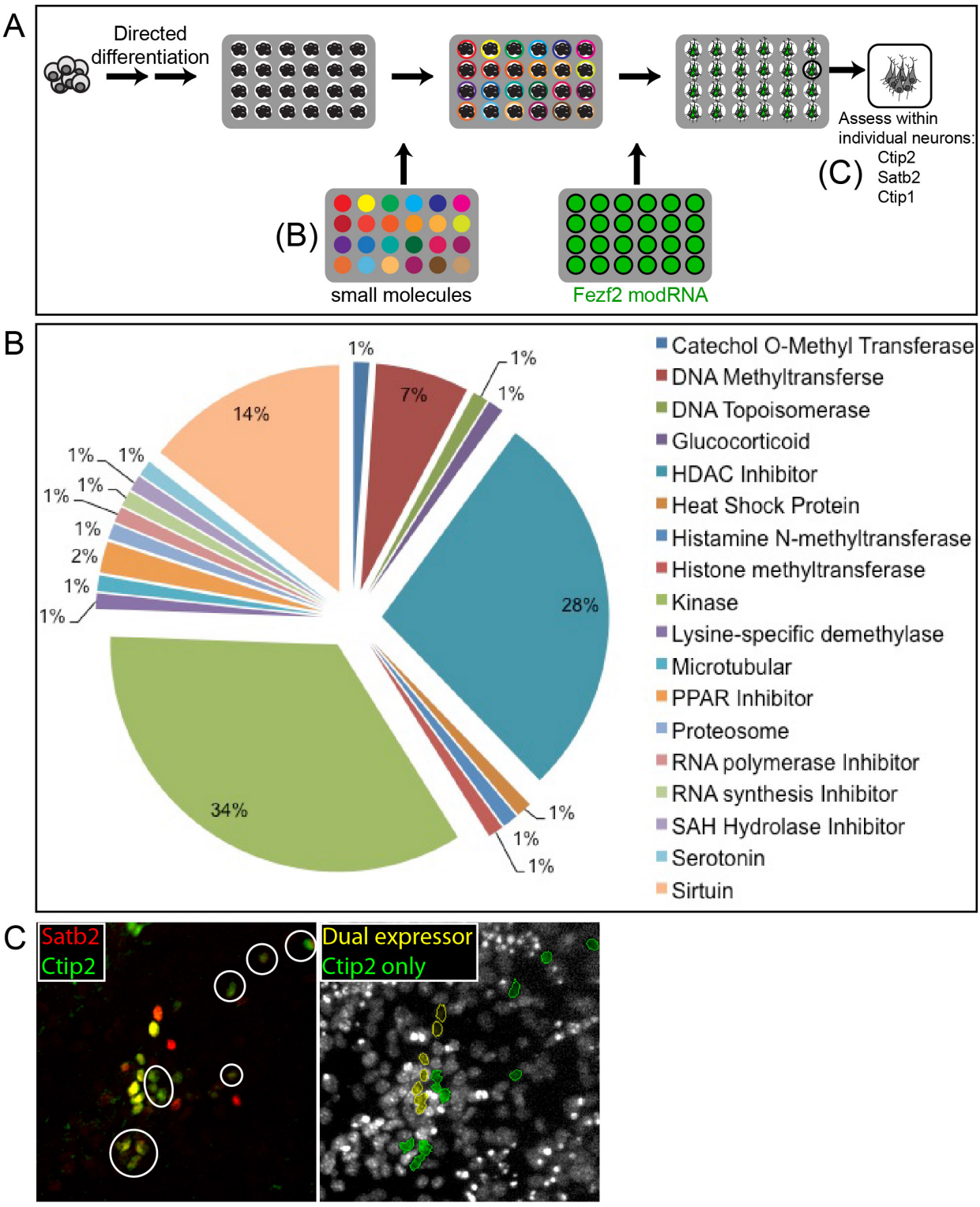
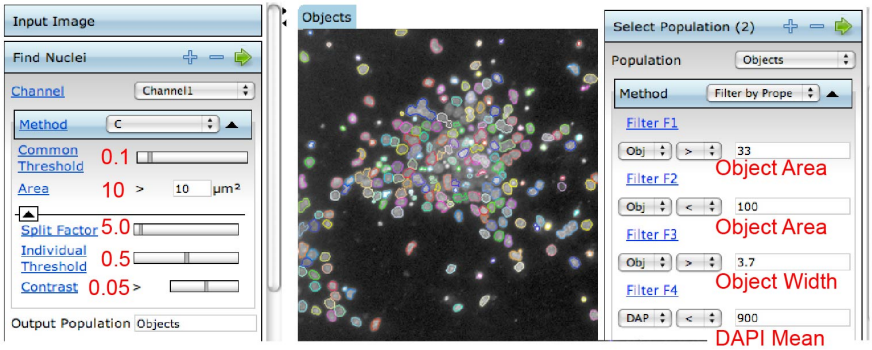
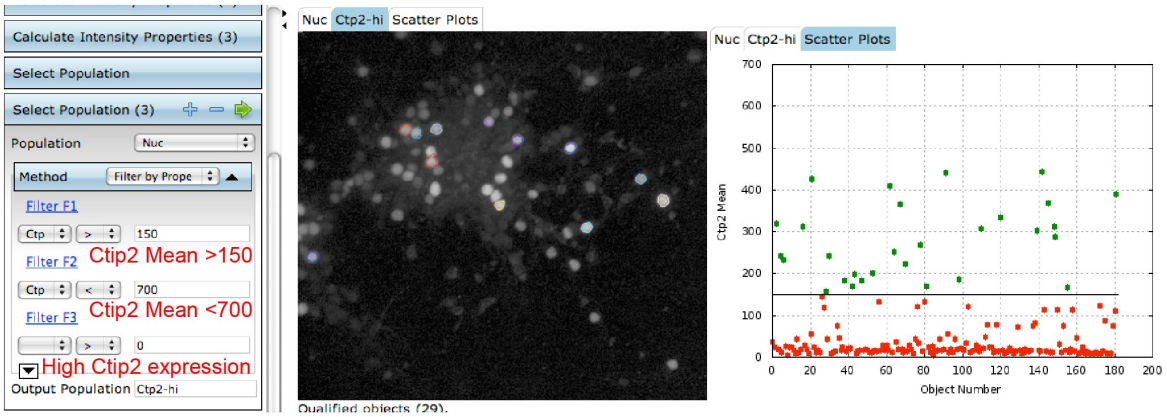


Figure 3.4 (Continued)

D Total Hoechst-positive nuclei (“NUC”)



Example of gating for high intensity, positive Ctip2 expression



Example of gating for low intensity, positive Satb2 expression

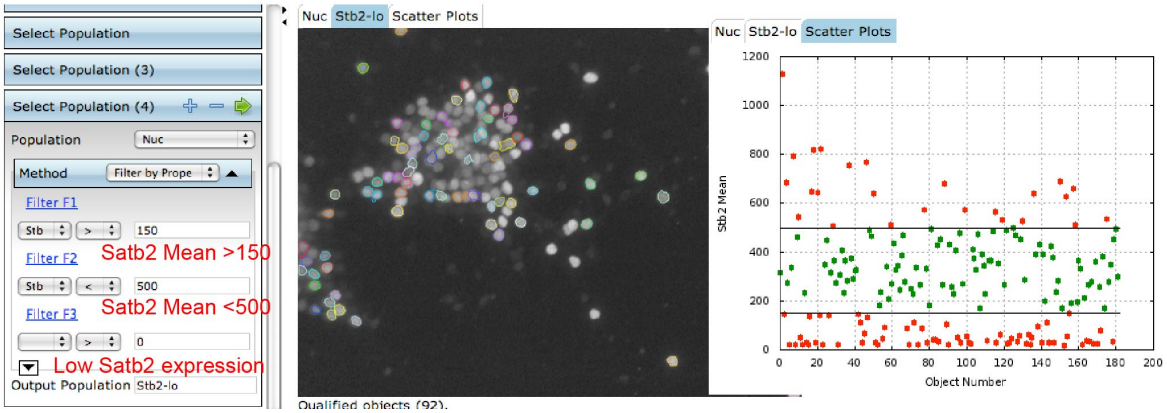


Figure 3.4 (Continued)

Figure 3.5. High-content screen of SCPN molecular refinement by ES-derived neurons identifies candidate small molecules, including Sirt1 inhibitor EX-527.

(A) Each well of a 96-well plate containing ES-derived neurons at 15 days was incubated with a distinct small molecule from the library (Figure 3.4B) for 96hrs, followed by transfection with GFP or Fezf2 modRNA, incubated for 48hrs, and finally fixed at 21 days *in vitro* (21DIV). Distinct small molecules enhanced or inhibited Fezf2-mediated SCPN subtype refinement, as indicated by the ratio of Ctip2(+)/Satb2(-) neurons to Ctip(+)/Satb2(+) neurons.

(B) Some candidate small molecules, from marked box in (A), increase the number of Ctip2-expressing neurons relative to Fezf2 induction alone.

(C) Fewer candidate small molecules, from marked box in (B), decrease the number of Satb2-expressing neurons relative to Fezf2 induction alone; these include Sirt1 inhibitor EX-527. Asterisks represent small molecules that modify HDAC Class III (Sirtuin). Black arrowheads represent GFP and Fezf2 transfection conditions, for comparison. Red arrowhead represents EX-527, a specific Sirt1 inhibitor. Data from this pilot experiment (N=1) are represented as a normalized ratio (approximately 1,000 cells per condition, from 40 randomly sampled fields at 20x magnification).

21DIV: 96hr small molecules, 48hr modRNA

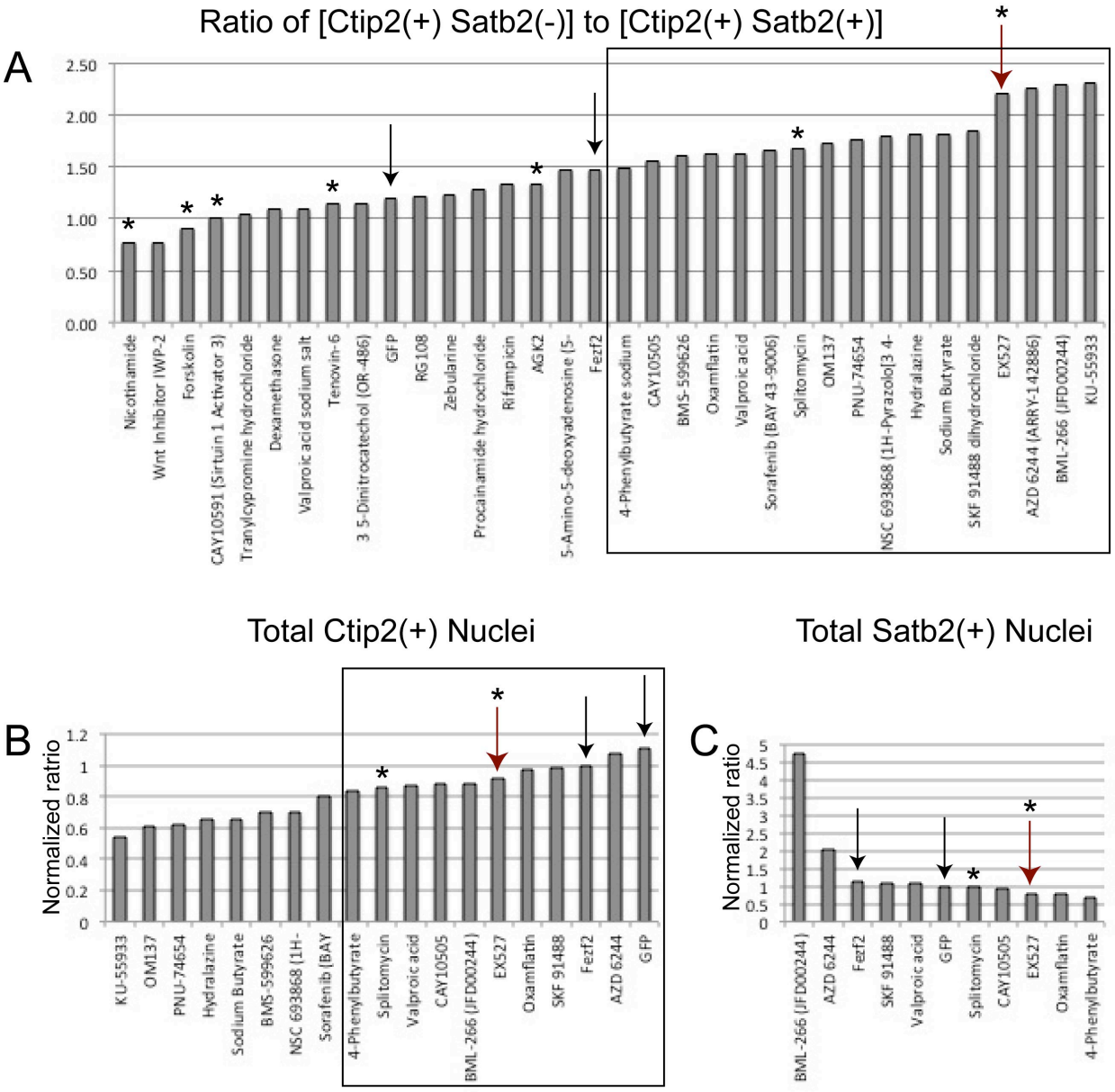


Figure 3.5 (Continued)

Based on these data, using multiple subtype markers and selection criteria, I selected Sirt1 as a target for subsequent analysis.

Sirt1 expression *in vivo* is CPN-subtype specific

Because *in vitro* ES-derived neocortical-like neurons are incompletely specified and maturation-stalled (**Chapter 2**), I next asked whether *in vivo* Sirt1 expression is consistent with the results of the screening approach in ES-derived neurons. I used immunocytochemistry to assess Sirt1 protein localization in the developing neocortex. While Sirt1 expression is broadly distributed in the brain, as previously reported (Hasegawa and Yoshikawa, 2008; Michan and Sinclair, 2007; Qin *et al.*, 2006), I hypothesized that its expression varies in distinct neocortical subtypes. Strikingly, I found that Sirt1 is differentially expressed in CPN and SCPN neocortical subtypes *in vivo* (**Figure 3.6**). Using wide-field fluorescence imaging on brain sections at P4, I found that Sirt1 is expressed throughout the rostro-caudal extent of the neocortex, in layers 2/3, 6, and subplate (**Figure 3.6A**). Using confocal imaging, I found that, as early as E18.5, Sirt1 expression is subtype-specific, with near-complete co-localization with Satb2-expressing CPN and relative exclusion by Ctip2-expressing SCPN in layer V (**Figure 3.6B**). Again at P4, confocal imaging shows that Sirt1 expression is excluded from increasingly mature SCPN (**Figure 3.6C**).

Figure 3.6. Sirt1 is differentially expressed by CPN and SCPN *in vivo*. (A) At P4, immuno-labeling indicates that Sirt1 is expressed along the entire rostral-caudal extent of the neocortex, in layers II/III, VI, and subplate (50um coronal section, wide-field fluorescence imaging). (B) At E18.5, Sirt1 expression in deep layers of motor cortex is mostly restricted to Satb2-expressing neurons and is absent or relatively low in Ctip2-expressing neurons (50um coronal section, confocal fluorescence imaging). (C) At P4, Sirt1 expression in deep layers of motor cortex is almost completely restricted to Satb2-expressing neurons (50um coronal section, confocal fluorescence imaging)

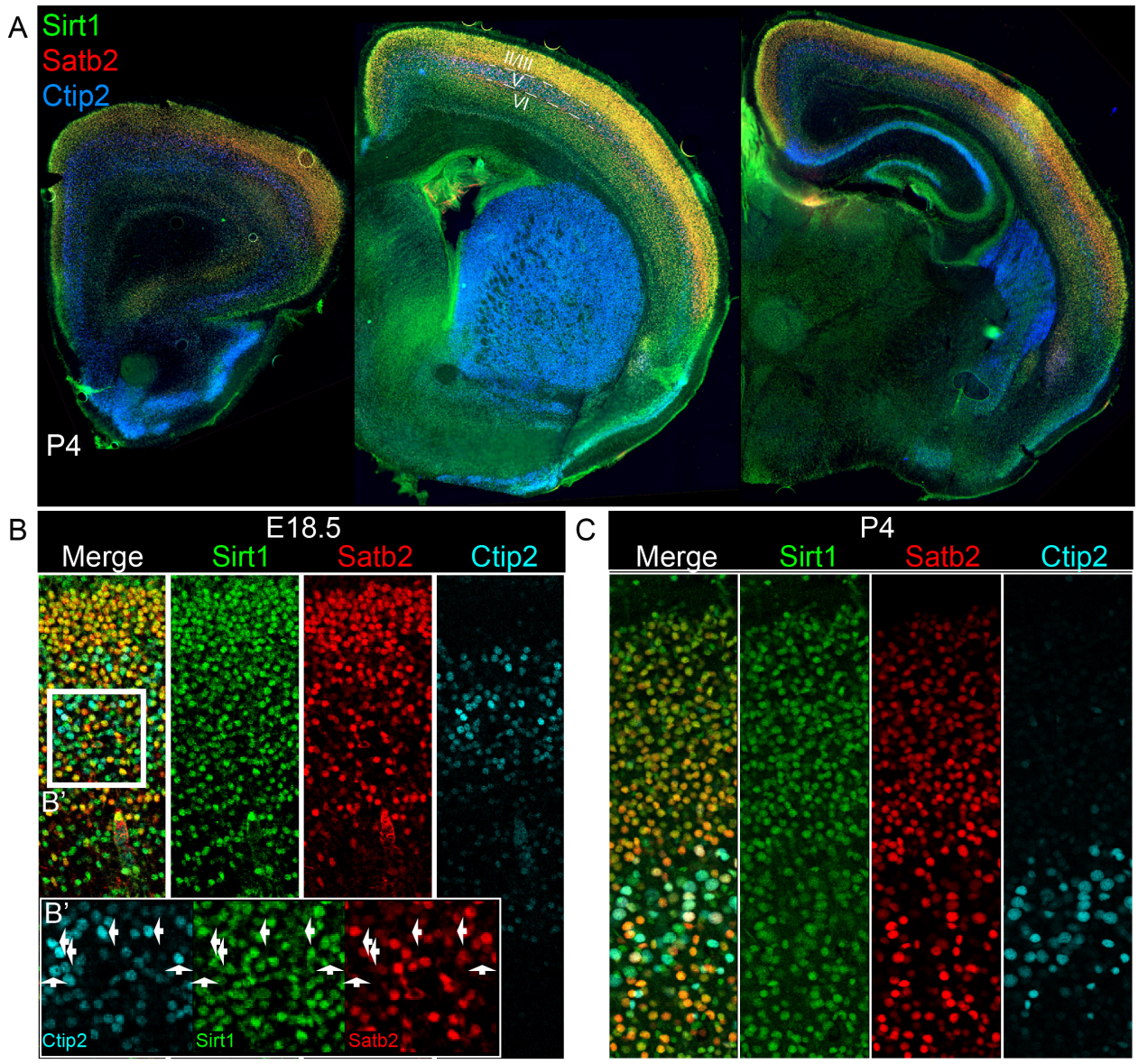


Figure 3.6 (Continued)

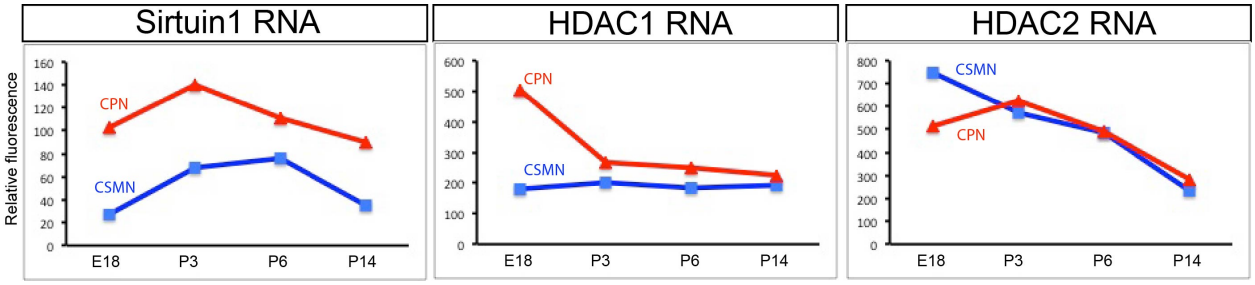


Figure 3.7: Sirt1 mRNA expression is CPN-specific in the neocortex at late embryonic and post-natal ages. (A) Sirt1 expression is higher in CPN (red lines) than in CSMN (blue lines) at E18.5, P3, P6, and P14; these populations were retrogradely labeled and isolated for comparative gene expression analysis at each time-point (data from Arlotta *et al.*, 2005). (B) Other HDACs (*e.g.*, HDAC1 and HDAC2) are not differentially expressed at all ages, using the same array data.

I next asked whether Sirt1 is differentially transcribed in pure populations of retrograde-labeled CPN versus corticospinal motor neurons (CSMN: a subtype of SCPN in layer V that project their axons to the spinal cord). The Macklis laboratory previously published microarray-based comparative gene expression analysis of retrograde-labeled CPN and CSMN (Molyneaux *et al.*, 2005). Using these data, I find that Sirt1 is the only differentially expressed histone deacetylase (HDAC) throughout post-mitotic neocortical differentiation at E18.5, P3, P6, and P14, with highest expression by CPN at all ages (**Figure 3.7**). Combined with the protein expression data in **Figure 3.6**, these findings show that Sirt1 protein and mRNA are specifically and highly expressed by Satb2-expressing CPN subtypes during corticogenesis, and are expressed at markedly lower levels by Ctip2-expressing SCPN, coinciding with all stages of subtype identity refinement. These *in vivo* findings support the CFuPN-specificity of Sirt1 from the screening approach in ES-derived neurons.

Sirt1 inhibition refines E12.5 SCPN subtype identity

Because ES-derived neocortical-like neurons have specific deficits in differentiation, and do not fully resemble primary neocortical neurons (**Chapter 2**), I next asked whether Sirt1 inhibition also promotes SCPN subtype distinction within *bonafide* primary neocortical neurons. In this experiment, Fezf2 was not induced with modRNA, because Fezf2 is already highly expressed by primary SCPN progenitors. Dissociated E12.5 neocortical neurons were treated with small molecule inhibitors of Sirt1 for six days (compounds were dissolved in DMSO; importantly, experimental control samples were treated with matched volumes of DMSO, considering its reported enhancement of terminal differentiation *in vitro* in Chetty *et al.*, 2013). I again identify EX-527, and a more specific Sirt1 inhibitor, CHIC-35, as potent factors that increase Ctip2/Satb2 subtype distinction (**Figure 3.8A**). CHIC-35 is Sirt1-specific with a

binding site within the Sirt1 catalytic cleft that blocks substrate binding (Napper *et al.*, 2005; Zhao *et al.*, 2013). Compared to non-specific inhibitors, both EX-527 and CHIC-35 show highly selective enhancement of Ctip2 refinement (**Figure 3.8A**).

To help determine whether Sirt1 inhibition broadly regulates SCPN subtype identity rather than only reducing Satb2 expression, I asked whether other subtype-specific refinements occur. Ctip1 is a transcription factor that regulates both subtype- and area-specific identity. Despite its close homology to Ctip2, Ctip1 is initially co-expressed with Ctip2, but its expression later becomes restricted to CPN (Woodworth *et al.*, unpublished data, 2013). Importantly, I find that Sirt1 small molecule inhibition promotes Ctip2/Ctip1 subtype distinction (**Figure 3.8B**). Based on both Ctip2/Satb2 and Ctip2/Ctip1 subtype distinction in the context of Fezf2 expression, these data indicate that Sirt1 inhibition refines neocortical subtype identity toward SCPN.

Given the roles of Sirt1 in neural progenitor differentiation and neuronal survival (Prozorovski *et al.*, 2008; Li *et al.*, 2008; Tiberi *et al.*, 2012; Hisahara *et al.*, 2008), I next tested an alternative hypothesis that Sirt1 inhibition potentially alters the proportions of progenitors and neurons, giving an impression of post-mitotic subtype distinction while instead acting at the progenitor level. To the contrary, I find that the increase in proportion of Ctip2-expressing neurons is nearly completely compensated by the loss of Ctip2/Satb2 dual-expressing neurons (**Figure 3.8A,B**). Because the combined fraction of Ctip2- and Satb2-expressing neurons remains constant, it is unlikely that the subtype refinement phenotype is attributable to changes in the proliferation of neocortical progenitors.

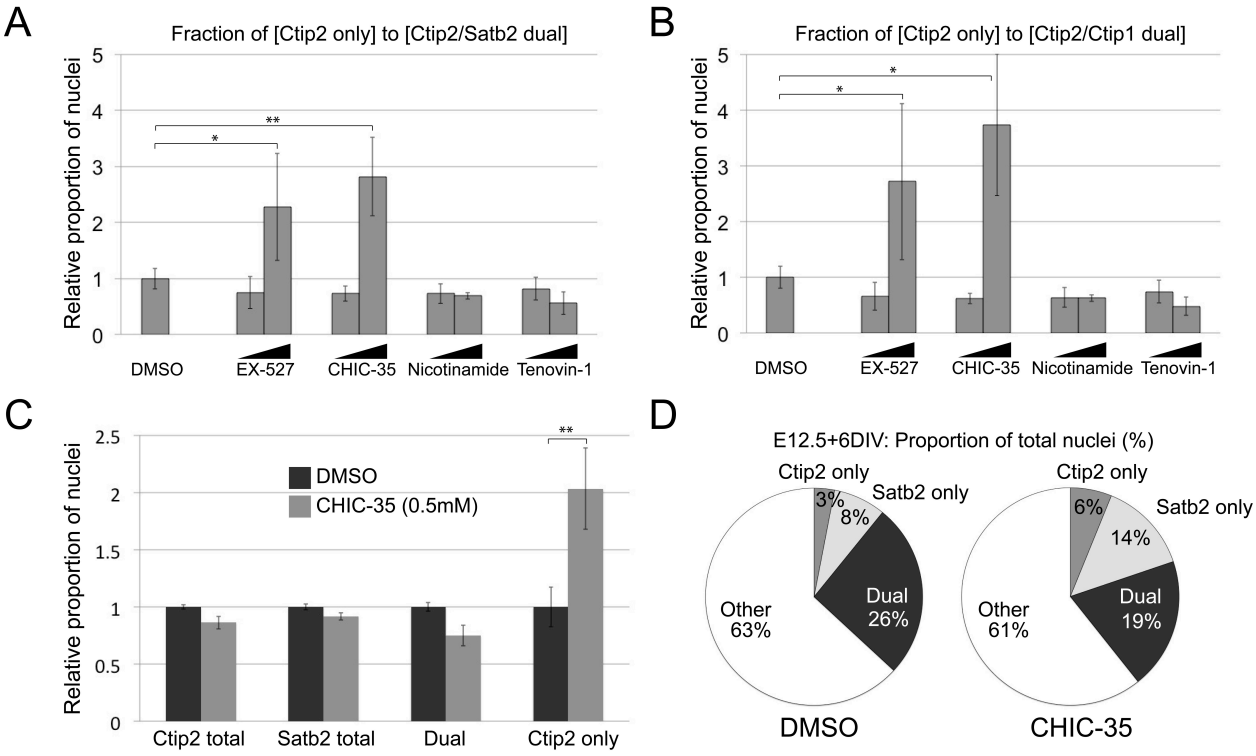


Figure 3.8. Sirt1 inhibition in dissociated E12.5 neocortical neurons enhances CFuPN subtype refinement, increasing the number of Ctip2-expressing neurons at the expense of Ctip2/Satb2 dual expressing neurons. (A) EX-527 and a more specific Sirt1 inhibitor, CHIC-35, increase Ctip2/Satb2 subtype distinction relative to DMSO-only controls (0.5uM and 5uM). (B) The same trend holds for Ctip2/Ctip1 subtype distinction. (C) Although the relative proportions to DMSO control of total Ctip2- and total Satb2-expressing neurons do not change following the CHIC-35 Sirt1 inhibition, the relative proportion of Ctip2/Satb2 dual-expressing neurons decreases. In contrast, the relative proportion of [Ctip2(+)/Satb2(-)] neurons increases. (D) Pie chart schematics depict the relative proportions to total nuclei of Ctip2-only neurons, Satb2-only neurons, Ctip2/Satb2-dual neurons, and unlabeled cells derived from E12.5 neocortical cells in the DMSO control versus CHIC-35 Sirt1 inhibition treatment conditions. Data are presented as mean +/- s.e.m. (N=3; >10,000 nuclei screened per condition from 60 randomly sampled fields at 20x magnification).) *P < 0.05; **P < 0.01 (unpaired t-test).

Although EX-527 and CHIC-35 are highly specific small molecule inhibitors of Sirt1 (Zhao *et al.*, 2013), to increase confidence that Sirt1 is the main target of repression, I pursued Sirt1-specific molecular knockdown with siRNA. I find that Sirt1 knockdown recapitulates the effect of small molecule inhibition of Sirt1, increasing both Ctip2/Satb2 and Ctip2/Ctip1 subtype specific refinement (**Figure 3.9**). These results further support the conclusion that Sirt1 inhibition enhances subtype specification toward SCPN.

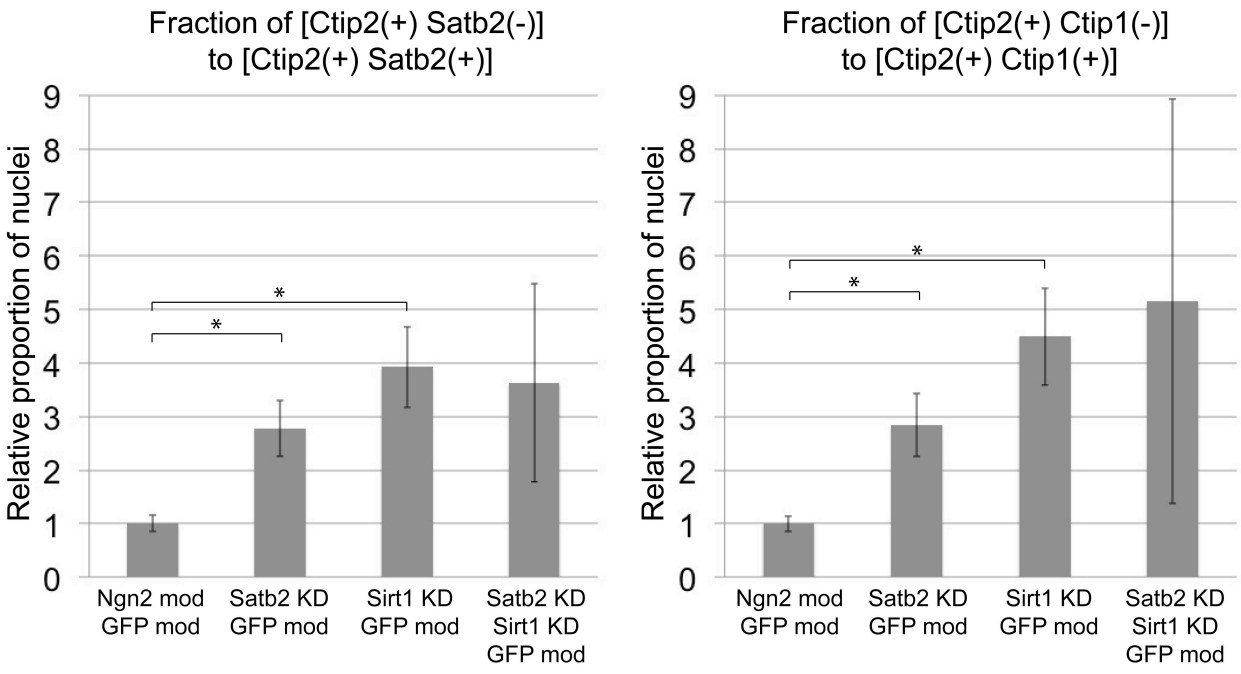


Figure 3.9: siRNA knockdown of Sirt1 recapitulates the effect of small molecule inhibition of Sirt1. (A) Ctip2/Satb2 refinement increases with Sirt1 knockdown. (B) Consistent with multiple molecular refinements during neocortical projection neuron subtype distinction, Ctip2/Ctip1 refinement also increases with Sirt1 knockdown. Data are presented as mean +/- s.e.m. (N=3; >10,000 nuclei screened per condition, from 60 randomly sampled fields at 20x magnification).) *P < 0.05 (unpaired t-test).

3.4 Discussion

I identify Sirt1 as the first chromatin-remodeling enzyme that is differentially expressed by two neocortical projection neuron subtypes: low in subcerebral projection neurons (SCPN), and high in inter-hemispheric callosal projection neurons (CPN). Sirt1 is a widely expressed NAD-dependent histone deacetylase (HDAC) with context-dependent roles in multiple cell types (Michan and Sinclair, 2007; Haigis and Sinclair, 2010; Gräff and Tsai, 2013). Although previous studies have shown a layer-specific Sirt1 expression pattern in the neocortex at E14.5 and adult ages (10 months post-natal), specific neocortical subtypes were not assessed (Hasegawa and Yoshikawa, 2008; Michan and Sinclair, 2007; Qin *et al.*, 2006). Using multiple markers of neocortical subtypes *in vivo*, I find that neocortical SCPN specifically exclude Sirt1 expression in mid- to late-corticogenesis, whereas Sirt1 is highly expressed by both deep and superficial layer CPN (**Figure 3.6**). This protein localization is verified by differential Sirt1 mRNA transcription in retrograde-labeled CSMN and CPN at post-mitotic stages of subtype identity refinement (**Figure 3.7**; Molyneaux *et al.*, 2005). In contrast, multiple other HDAC enzymes do not show projection neuron subtype specificity in the neocortex (**Figure 3.7**; Molyneaux *et al.*, 2005; MacDonald and Roskams, 2008).

The experimental screening approach used to identify Sirt1 was based on the hypothesis that early chromatin remodeling might enable Fezf2-mediated induction of SCPN identity within incompletely specified ES-derived neocortical progenitors. In support of this approach of “epigenetic priming” (Tursun *et al*., 2011; Patel *et al*., 2012; Gräff and Tsai, 2013; Papp and Plath, 2013), multiple lines of evidence indicate that Fezf2-mediated SCPN identity refinement (*in vivo* and in ES-derived neurons) requires a permissive molecular context during differentiation. First, transient Fezf2 expression is sufficient to generate SCPN in Fezf2-null mice at E12.5, and not at E15.5 (**Figure 3.1**). At later ages (E13.5 through P7), a higher dose or

duration of Fezf2 expression can re-specify alternate neocortical subtypes to SCPN identity (Molyneaux *et al.*, 2005; Chen *et al.*, 2008; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013). However, after E15.5, the mis-expression of Fezf2 does not induce Ctip2 expression in most neurons (Chen *et al.*, 2008; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013), and does not repress Satb2 expression (Chen *et al.*, 2008; De la Rossa *et al.*, 2013). Together, these findings indicate that an optimal molecular context exists for the Fezf2-mediated specification of SCPN, induction of Ctip2 expression, and stable epigenetic silencing of Satb2 at approximately E12.5.

These *in vivo* data emphasize the required specificity in the timing of Fezf2 expression and the importance of an optimal neocortical molecular context *in vitro* for Fezf2 to direct differentiation of ES-derived neurons. Fezf2 induction prior to neocortical differentiation among non-neocortical-like neurons does not induce SCPN identity (Wang *et al.*, 2011; Sadegh, unpublished data, 2011). Moreover, when Fezf2 modRNA is induced at a time approximating E12.5 neocortical differentiation, it does not significantly increase SCPN subtype-specific transcription factor expression within ES-derived neocortical cells (**Figure 3.3**). These *in vitro* data indicate that, although timing is important, it is not sufficient to enable complete Fezf2-mediated SCPN differentiation; incompletely specified and maturation-stalled ES-derived neocortical neurons are not an optimal molecular context (**Chapter 2**). Therefore, to enhance the context of Fezf2-directed differentiation, I pursued a high content screening approach to identify small molecule modifiers of chromatin structure.

To identify a specific chromatin-remodeling enzyme that can enable Fezf2-mediated SCPN differentiation within maturation-stalled ES-derived neocortical cells, I screened diverse types of epigenetic modifiers (**Figure 3.4**). I then induced ES-derived cells with Fezf2 and assessed the extent of neocortical subtype identity refinement with multiple markers. From the initial pilot screening experiment **(Figure 3.5)**, I find that the inhibition of Sirt1 preceding Fezf2

induction enhances SCPN identity refinement in ES-derived neocortical neurons. Within primary neocortical cells, I show that direct Sirt1 inhibition, by either small molecule or knockdown approaches in primary neocortical neurons, promotes mature molecular refinement of Fezf2-mediated SCPN identity (**Figure 3.8, 3.9**). These findings are consistent with SCPN subtype-specific Sirt1 exclusion (**Figure 3.6**), which temporally correlates with heightened post-mitotic Fezf2 expression. The data presented support the interpretation that Sirt1 down-regulation is functionally important for the refinement of SCPN identity *in vivo* and useful for the refinement of ES-derived SCPN *in vitro*.

Although Sirt1-null mice have not yet been assessed for subtype-specific deficits in the neocortex, their gross neocortical anatomy (*e.g.* intact corpus callosum, absence of Probst bundles) appears intact (Cheng *et al.*, 2003; McBurney *et al.*, 2003; Michán *et al.*, 2010), suggesting that Sirt1 is not required for CPN specification. Given that subtle subtype identity deficits might exist in Sirt1-null mice, any interpretations must take into account the multiple known functions of Sirt1 in neocortical differentiation. At a stage prior to subtype refinement, Sirt1 regulates neurogenesis within neocortical progenitors; Sirt1 is recruited to repress the Notch-Hes pathway to irreversibly promote neurogenesis despite persistent Notch signaling (Tiberi *et al.*, 2012; Hisahara *et al.*, 2008). Moreover, other critical chromatin modifiers theoretically required for CPN differentiation might compensate for Sirt1 loss of function by modifying the global chromatin state.

CPN are an evolutionarily more recent and diversified subtype of neocortical neurons, and likely employ multiple sequential epigenetic mechanisms in their specification, molecular refinement, and maturation (Molyneaux *et al.*, 2009; MacDonald and Roskams, 2009; Kishi and Macklis, 2010; Fame *et al.*, 2011). At late stages of maturation of layer 2/3 CPN (*e.g.* eight postnatal weeks), the widely expressed methyl binding protein MeCP2 is required for the

development and/or maintenance of dendritic complexity and soma size (Kishi and Macklis, 2004, 2010). At earlier stages of CPN development, Satb2 is a required CPN-specific transcription factor that indirectly guides chromatin remodeling by binding to matrix attachment regions (MAR) and recruiting HDAC enzymes through a binding partner, Ski (Britanova *et al.*, 2005; Britanova *et al.*, 2008; Alcamo *et al.*, 2008; Gyorgy *et al.*, 2008; Baranek *et al.*, 2012). Together, with varying extents of CPN-specificity, Sirt1, Satb2/Ski, and MeCP2 might coordinate chromatin remodeling in CPN at distinct stages of development.

The subtype-specificity of Sirt1 expression in the neocortex is also interesting because Sirt1 is implicated in the oxidative stress response and survival of neurons (Li *et al.*, 2008; Prozorovski *et al.*, 2008). This suggests that Sirt1-expressing CPN might be resistant to metabolic insults; conversely, it raises the possibility that reduced Sirt1 expression might increase SCPN sensitivity to metabolic stress and oxidizing conditions. SCPN and the subpopulation of corticospinal motor neurons are the neocortical neurons that selectively degenerate in amyotrophic lateral sclerosis (ALS; Ozdinler and Macklis, 2006; Zang and Cheema, 2002). In fact, Sirt1 over-expression has been shown to promote short-term neuronal survival in dissociated neocortical cells mis-expressing ALS associated mutant SOD1 (Kim *et al.*, 2007). More broadly, non-specific HDAC inhibitors show neuro-protective properties in mouse models of ALS (Petri *et al.*, 2006; Rouaux *et al.*, 2007). Although highly speculative, these findings are consistent with SCPN vulnerability to metabolic stress. Given the multiple roles of Sirt1 in other cell populations, the full implications of its subtype specificity in the neocortex remain to be explored.

Together, these findings strongly suggest a stage-specific role for Sirt1 in the differential molecular refinement of neocortical SCPN and CPN subtype identity. Despite widespread expression of Sirt1, these data implicate a subtype-specific mechanism of

regulation in neocortical projection neurons. Importantly, these findings demonstrate the utility of combining epigenetic priming with subtype-specific transcription factor induction in ES cell directed differentiation. This strategy provides a specific enhancement to protocols of directed SCPN differentiation.

3.5 Experimental Procedures

Cell culture and differentiation

Murine embryonic stem cells: Feeder-free E14Tg2a (Baygenomics) mouse embryonic stem cells were propagated using standard procedures (Ying *et al.*, 2003) on gelatin-coated (0.1% gelatin, StemCell Technologies) cell culture treated plastic dishes. Mouse embryonic stem cell media is GMEM (Invitrogen) supplemented with 10% ESC-certified fetal bovine serum (vol/vol, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM β-mercaptoethanol (Sigma), 50 U/mL penicillin/streptomycin and 1000 U/mL leukemia inhibitor factor (ESGRO).

For differentiation, ES cells were plated at low density (5,000 cells/cm^2) on gelatin-coated plastic dishes in ES cell medium, and cultured as described (Gaspard *et al.*, 2009). Briefly, ESCs were trypsinized, dissociated, and plated on gelatin-coated cell culture plates. Medium was changed to DDM after one day. DDM consists of DMEM/F12 (Invitrogen-Gibco) supplemented with N2 supplement (N2 supplement consists of 8.61uM insulin, 1mM transferrin, 2uM progesterone, 10mM putrescine and 3uM selenite; Invitrogen-Gibco), 2mM glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 0.5 mg/ml bovine serum albumin fraction V (all from Invitrogen-Gibco), and 0.1mM β-mercaptoethanol (Sigma).

Cyclopamine (Calbiochem) was added from day 2 to day 10 in the differentiation medium at a final concentration of 1uM. After 10 to 14 days of differentiation, cells were trypsinized, dissociated and plated on poly-lysine/laminin (Becton-Dickinson) coated glass coverslips and allowed to grow for 4–14 days in N2B27 medium. N2B27 medium consists of a 1:1 mixture of DDM and Neurobasal that is supplemented with B27 (without vitamin A; Invitrogen-Gibco) and 2 mM glutamine.

Mice

All mice used in these experiments were handled according to guidelines of the National Institutes of Health (NIH), and all procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of Harvard University. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Fezf2-null mice were the generous gift of S. McConnell (Chen *et al.*, 2005). Wild-type CD1 mice were used in all other experiments (Charles River Laboratories).

Immunocytochemistry

Mice were deeply anesthetized with a lethal dose of anesthetic (Avertin) or hypothermia, and perfused trans-cardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde, rinsed with PBS, and sectioned coronally with 50μm thickness on a vibrating microtome (Leica). Sections were blocked in 1% BSA (Sigma), and 0.3% Triton X-100 (Sigma) for 1hr at room temperature before incubation with primary antibodies overnight at 4°C. Primary antibodies and dilutions used were: rat antibody to Ctip2 (1:500, Abcam), mouse antibody to Satb2 (1:200, Abcam), rabbit antibody to Sirt1 (1:250, Millipore), rabbit antibody to Ctip1 (1:500, Abcam), rabbit antibody to GFP (1:500, Invitrogen), chicken antibody to Nestin (1:500, Novus Biologicals), mouse antibody to TuJ (1:500, Covance), mouse antibody to Map2 (1:500, Sigma), and mouse antibody to NeuN (1:250, Millipore). Alexa fluorophore conjugated secondary antibodies from Invitrogen were used at a dilution of 1:1000. Hoechst 33342 counterstain was used to visualize nuclei (1:3,000, Invitrogen).

Visualization and Analysis

Wide-field image acquisition was performed using a Nikon 90i epifluorescence microscope with a Clara DR-328G cooled CCD digital camera (Andor Technology, Belfast, Northern Ireland). Confocal imaging was performed with a BioRad Radiance 2100 Rainbow laser-scanning confocal microscope based on a Nikon E800 microscope. Images were assembled in Adobe Photoshop and Illustrator (CS3, CS5), with adjustments for contrast, brightness, and color balance to obtain optimal visual reproduction of data.

High-content small molecule screening

High content screening protocol was adapted from (Makhortova *et al.*, 2011). Briefly, ES cells were seeded at 5,000 cells per well in 96-well plates and treated in duplicate at 10 μM, 1 μM and 0.1 μM with individual compounds from the screening library, a custom set of 80 chemicals affecting histone deacetylases, methyltransferases, and kinases (**Figure 3.4**). These compounds were curated from the following chemical libraries: LOPAC1280 Collection (Sigma-Aldrich), Spectrum Collection (Microsource Discovery Systems), Prestwick Chemical Library. EX-527 (Sigma), CHIC-35 (Sigma), and nicotinamide (Sigma) were re-suspended in DMSO, according to the manufacturer's instructions.

Following immunostaining, 96-well plates were scanned by an automated confocal microscope (PerkinElmer Opera) at 20X magnification with separate fluorescence exposures from a UV light source and 488, 546, 647nM lasers. Image analysis was done using Columbus software (version 2.3.0; PerkinElmer; see also Figure 3.4C,D), which automatically set the boundaries of cell nuclei based on Hoechst staining. These boundaries were optimized by manual inspection to exclude nuclear fragments or adjacent double nuclei based on the total area and staining intensity of Hoechst-positive nuclei. Next, the intensity of antibody staining

for each distinct transcription factor in each nucleus was quantified. A high threshold for positive antibody staining was manually established compared to baseline staining without primary antibody, for Ctip2, Satb2, and Ctip1. Relatively high thresholds of positivity were established because populations of ES-derived neurons expressed a continuum of transcription factor staining intensities, in contrast to populations of primary dissociated E15.5 neocortical neurons, which displayed typically bimodal staining (low, high).

RNA synthesis and transfection

ModRNA was generated as per Warren et al. (2010). Briefly, RNA was synthesized with the MEGAscript T7 kit (Ambion, Austin, TX). A custom ribonucleoside blend was used comprising 6 mM 5' cap analog (New England Biolabs), 7.5 mM adenosine triphosphate and 1.5 mM guanosine triphosphate (USB, Cleveland, OH), 7.5 mM 5-methylcytidine triphosphate and 7.5 mM pseudo-uridine triphosphate (TriLink Biotechnologies, San Diego, CA). Reactions were incubated 3–6 hr at 37°C and DNase treated as directed by the manufacturer. RNA was purified with Ambion MEGAclear spin columns, then treated with Antarctic Phosphatase (New England Biolabs) for 30 min at 37°C to remove residual 5' triphosphates. Treated RNA was re-purified, quantitated by Nano-drop (Thermo), and adjusted to 100ng/mL working concentration by addition of Tris-EDTA (pH 7.0). Transfections of modRNA and multiple siRNA targeted against Sirt1 and Satb2 (both from Santa Cruz) were carried out with RNAiMAX (Invitrogen), as per the manufacturer's instructions.

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Chapter 4

Sequential and transient expression of specific developmental transcriptional regulators more specifically directs corticofugal projection neuron differentiation from mouse embryonic stem cells

Publication

This chapter is in the process of being prepared for manuscript submission in the fall of 2013. Experiments are ongoing.

Sadegh, C., Ebina, W., Rossi, D. and Macklis, J.D. “Sequential and transient expression of specific developmental transcriptional regulators more specifically directs corticofugal projection neuron differentiation from mouse ES cells.”

Author contributions

I initiated the project independently. Wataru Ebina in Derrick Rossi’s laboratory provided expertise for the application of synthetic modified mRNA technology, reagent support, and discussion for this work. Data from this chapter is not incorporated into his dissertation research.

4.1 Abstract

During development of the neocortex, many diverse projection neuron subtypes are generated under the relatively precise regulation of sequentially and transiently expressed molecular controls, centrally including transcriptional regulators. One major class of projection neurons, corticofugal projection neurons (CFuPN), sends axons to sub-cortical targets including thalamus, striatum, brainstem, and spinal cord. In recent years, a growing number of stage-specific transcriptional regulators that act sequentially to direct the progressive specification and differentiation of CFuPN have been identified.

Existing protocols to generate neocortical-like neurons from embryonic stem (ES) cells are not directed toward the generation of specific classes, types, or subtypes of projection neurons; instead, an established monolayer protocol of ES cell differentiation relies on spontaneous generation of neocortical-like neurons, which I have shown to be heterogeneous and maturation-stalled (**Chapter 2**).

By comparison to *in vivo* CFuPN development, I investigated whether the sequential and transient expression of "nested" forebrain, neocortex, and CFuPN-specific transcriptional regulators can progressively direct ES cells to acquire CFuPN identity *in vitro*. We applied synthetic modified mRNAs (modRNA), which are *in vitro* transcribed messenger RNAs with specific nucleotide substitutions to avoid innate cellular immune responses to foreign single-strand RNA; modRNA have been demonstrated to powerfully function with a transient temporal course and controllable dosage of expression (Warren *et al.*, 2010).

Using modRNA to mimic the timing and dosage of gene expression during development, I show that sequential expression of twelve progressively CFuPN-specific transcription factors accelerates neurogenesis toward CFuPN phenotype *in vitro*. When micro-transplanted into postnatal neocortex, these ES-derived, CFuPN-directed neurons

appropriately project their axons to ipsilateral thalamic and midbrain targets, and avoid inappropriate projections to the contralateral hemisphere. I tested rigorously for cell fusion with host CFuPN and find no evidence for it, based on numerous criteria, including: 1) progressive axon extension over the course of six days; 2) the corticofugal specificity of axonal projections; and 3) the relatively superficial position of transplanted neurons relative to host CFuPN. Together, these data provide evidence that developmentally informed molecular "programming" of ES-derived progenitors can direct and enhance their differentiation into desired neocortical neuron classes and subtypes.

These results demonstrate the feasibility of sequentially and transiently expressing "nested" molecular controls over CFuPN development to direct the differentiation of one broad class of neocortical projection neurons. Refinements of this initial approach might enable increasingly specific directed differentiation of other classes, types, and subtypes of neocortical neurons.

4.2 Introduction

Corticofugal projection neurons (CFuPN) are a broad class of neocortical neurons that send axonal projections from the neocortex to distal targets in the thalamus, midbrain, hindbrain, and spinal cord (Molyneaux *et al.*, 2007). The recent delineation of progressive stages of neocortical and CFuPN subtype differentiation (Azim *et al.*, 2009; Woodworth *et al.*, 2012) now makes it possible to raise the question of whether a simplified sequence of "nested" transcriptional regulators (Custo Greig *et al.*, in editorial revision, 2013) might be sufficient to direct the differentiation of embryonic stem (ES) cells into CFuPN.

As first exemplified by the generation of ES-derived spinal motor neurons (SMN), replicating appropriate levels of key morphogens associated with defined rostral-caudal and dorsal-ventral domains *in vivo* can be used to direct ES cell differentiation (Wichterle *et al.*, 2002). These regionally-specified ES-derived ventral spinal cord progenitors spontaneously give rise to heterogeneous motor column subclasses of SMN (Peljto and Wichterle, 2011).

Multiple related protocols have modified this morphogen-based approach to instead direct rostral and dorsal differentiation to generate progenitors with pallial telencephalic characteristics; these ES-derived, pallial-like progenitors spontaneously generate highly heterogeneous neocortical-like neurons *in vitro* (Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Mariani *et al.*, 2012; Shi *et al.*, 2012; Nasu *et al.*, 2012; Espuny-Camacho *et al.*, 2013; **Chapter 2**), potentially including mixed populations of sub-cortical CFuPN and inter-hemispheric callosal projection neurons (CPN), as assessed by bulk transplantation experiments (Gaspard *et al.*, 2008; Espuny-Camacho *et al.*, 2013).

The spontaneous differentiation of ES-derived pallial progenitors recapitulates some, but not all, aspects of corticogenesis (Hansen *et al.*, 2011). In particular, ES-derived neocortical progenitors and neurons, including CFuPN-like neurons (*e.g.*, Ctip2 positive / Satb2 negative),

are incompletely specified and maturation-stalled (**Chapter 2**). These deficits pose distinct challenges to the generation of fully specified CFuPN.

Several transcription factors are known to direct specification of CFuPN identity *in vivo*, but the expression of these controls *in vitro* has proved ineffective for CFuPN enrichment by ES-derived progenitors. For example, overexpression of CFuPN transcriptional regulators (*e.g.*, Fezf2, (Molyneaux *et al.*, 2005); Ctip2, (Chen *et al.*, 2008); and Fezf1, (Eckler *et al.*, 2011) can induce sub-cortical CFuPN specification in neocortical progenitors *in vivo*. The most potent of these genes, Fezf2, can additionally reprogram striatal medium spiny neurons, post-mitotic layer IV neurons, and post-mitotic callosal projection neurons to acquire multiple characteristics of CFuPN *in vivo* (Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013). In striking contrast to *in vivo* misexpression, Fezf2 does not direct CFuPN specification when expressed early in ES cell differentiation protocols (Wang *et al.*, 2011). However, I find that Fezf2, when expressed at later time points of ES cell differentiation, directs more mature CFuPN differentiation without increasing overall numbers of CFuPN (**Chapter 3**).

Because many of the deficits I have identified among spontaneously-generated neocortical-like neurons, from an established protocol of ES cell differentiation, precede the generation of neurons and occur at the progenitor level (**Chapter 2**), my data collectively suggest the need for a more comprehensive approach to faithfully direct differentiation of ES cells into CFuPN, beginning at earlier progenitor stages of differentiation. I hypothesized that closely approximating multiple stages of CFuPN development, including neural induction, pallial specification, and post-mitotic subtype identity refinement, might more effectively direct mouse ES cells toward CFuPN identity.

An emerging understanding of CFuPN differentiation by neocortical progenitors (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Azim *et al.*, 2009; Shim *et al.*, 2012) and post-mitotic

neocortical neurons (Weimann *et al.*, 1999; Arlotta *et al.*, 2005; Lai *et al.*, 2008; Joshi *et al.*, 2008; Azim *et al.*, 2009; Bedogni *et al.*, 2010; McKenna *et al.*, 2011) indicate a sequence of "nested" transcriptional regulators that might act synergistically to promote CFuPN differentiation (Woodworth *et al.*, 2012; Custo Greig *et al.*, unpublished data, 2013). Importantly, most of these developmental regulators are expressed transiently, sequentially, and with dose-dependence; these genes are only rarely maintained at later stages of development (*e.g.*, Fezf2 is expressed in adult mouse neocortex, (Inoue *et al.*, 2004; Molyneaux *et al.*, 2005; Ozdinler *et al.*, 2011).

To best approximate the transient and sequential nature of CFuPN molecular development, I decided to express critical developmental transcriptional regulators using synthetic modified mRNA (modRNA; Warren *et al.*, 2010). modRNA technology is especially well-suited for directed differentiation, as it enables greater precision for controlling the timing and dosage of critical transcriptional regulators; its transient and non-integrating characteristics make it suitable for short-duration, combinatorial, and stage-specific manipulations in a variety of cell types. In Chapter 3, I have previously demonstrated that Fezf2 expression by modRNA can direct CFuPN differentiation both *in vivo* and *in vitro* (**Chapter 3**).

In this chapter, I show that transient modRNA-based expression of twelve critical CFuPN transcription factors provided at five time points *in vitro* accelerates ES-derived neocortical neurogenesis, and directs differentiation of ES cells into CFuPN. When micro-transplanted into the neocortical parenchyma, these ES-derived neurons send ipsilateral axonal projections to thalamic and midbrain targets, and appropriately do not innervate the contralateral hemisphere, indicating that they have acquired corticofugal identity.

4.3 Results

Synthetic, modified mRNA enables time- and dose-dependent protein expression

Synthetic modified mRNAs (modRNA) are *in vitro* transcribed messenger RNAs with specific nucleotide substitutions to avoid innate cellular immune responses to foreign single-strand RNA (Warren *et al.*, 2010). The modRNA and expressed proteins are transient, normally degrading over 24-48hrs; sustained expression can be achieved with repeated daily transfection of cells, and expression levels are correlated with the dosage of transfected modRNA.

To investigate whether modRNA enables precise control over gene dosage and kinetics of expression in feeder-free E14Tg2a ES cell-derived neocortical-like progenitors, I first tested the transient expression of modRNA coding for GFP. Following cationic lipid-based modRNA transfection, GFP is expressed by 40-50% of total cells, and the intensity of native GFP expression is dose-dependent (0, 2, 4, 8ug modRNA; **Figure 3.1**). modRNA-induced GFP expression is also time-dependent, beginning expression as early as 3hrs, plateauing after 24hrs, and decreasing in intensity by 48hrs, consistent with the published time course (**Figure 3.1**).

To investigate whether modRNA transfection has cell type-specific tropism within feeder-free E14Tg2a ES cell-derived populations, I tested the expression of GFP modRNA specifically within progenitors or immature neurons. After 24hrs, GFP modRNA is expressed in both progenitors and neurons, as well as in other cells, without a discernible bias (**Figure 3.2**). Multiple modRNA are consistently expressed in the same cells (observed following co-transfection of GFP and mCherry modRNA; **Figure 3.2**).

Sequential expression of modRNA-encoded developmental transcriptional regulators enriches for ES-derived neurons

In order to pursue the strategy of directed differentiation by sequential and transient gene expression, we generated modRNA for each of twelve transcription factors selected on the basis of their critical functions during development (**Table 4.1**). Tau::GFP mouse ES cells (Wernig *et al.*, 2002) were dissociated at low density on gelatin-coated plastic dishes following a well-characterized adherent cell culture protocol for generating neocortical neurons, as previously described (Gaspard *et al.*, 2009; **Chapter 2**). Starting 24hr after initial plating, termed day 0, ES cells were given serum-free, defined differentiation medium (DDM) containing N2. These day 0 ES cells were immediately transfected with the first set of modRNA (Sox2, Lhx2, Pax6) to promote neural induction and pallial progenitor specification.

The transcription factors Sox2, Lhx2, and Pax6 were selected for the earliest expression in ES cell culture because of their well-established roles in neural, pallial, and neocortical specification. At the earliest stage of embryonic development, Sox2 acts to suppress mesendoderm fates in the mouse blastocyst and human pluripotent stem cells (Keramari *et al.*, 2010; Thomson *et al.*, 2011; Sarkar and Hochedlinger, 2013); Sox2 can also induce neural stem cell-like identity in fibroblasts (Ring *et al.*, 2012; Lujan *et al.*, 2012). Sox2 expression at E8.5 is required for proper pallial differentiation (Aota *et al.*, 2003; Götz *et al.*, 1998; reviewed in Georgala *et al.*, 2011). Lhx2 is also required for proper neocortical progenitor development at approximately E10, and, importantly, activates the expression of Hes1 in neocortical progenitors (Chou and O'Leary, 2013; Roy *et al.*, 2013).

Table 4.1. Expression of twelve critical transcription factors is "nested" within sequential progenitor and post-mitotic stages of CFuPN development. These transcription factors have required functions in at least five sequential, "nested" stages of development: pallial progenitors; neocortical progenitors; CFuPN progenitors; CFuPN during early corticogenesis; and CFuPN / SCPN refinement during late corticogenesis. Sets of transcription factors within each of these five stages are sequentially and transiently expressed by ES-derived populations using modRNA at 48hr intervals in an established monolayer protocol that generates pallial-like progenitors (Gaspard *et al.*, 2009).

"Nested" stages	Gene	Expression	Citation
Pallial, neocortical progenitors	Sox2	early neural progenitors, pallial progenitors	Keramari *et al.*, 2010; Thomson *et al.*, 2011
	Lhx2	neocortical progenitors	Chou and O'Leary, 2013; Roy *et al.*, 2013
	Pax6	pallial and neocortical progenitors	Manuel *et al.*, 2007; Berninger et al., 2007
Neocortical neurogenesis	Ngn2	neocortical progenitors and early post-mitotic neurons	Fode *et al.*, 2000; Schuurmans *et al.*, 2004
	NeuroD4	neocortical progenitors and early post-mitotic neurons	Mattar *et al.*, 2008
CFuPN progenitor specification	Sox11, Sox4	neocortical progenitors and post-mitotic neurons	Shim *et al.*, 2012
	Fezf2	potentially fate-restricted CFuPN progenitors and post-mitotic CFuPN	Molyneaux *et al.*, 2005; Chen *et al.*, 2005
CFuPN (early corticogenesis)	Ctip2	post-mitotic CFuPN; highest in SCPN	Arlotta *et al.*, 2005; Chen *et al.,* 2008
	Sox5	post-mitotic CFuPN; highest in CThPN	Lai *et al.*, 2008
	Tbr1	post-mitotic CFuPN; highest in CThPN	Bedogni *et al.*, 2010; McKenna *et al.*, 2011
CFuPN (late corticogenesis)	Fezf2	potentially fate-restricted CFuPN progenitors and post-mitotic CFuPN	Molyneaux *et al.*, 2005; Chen *et al.*, 2005
	Ctip2	post-mitotic CFuPN; highest in SCPN	Arlotta *et al.*, 2005; Chen *et al.,* 2008
	Otx1	post-mitotic SCPN	Weimann *et al.*, 1999

On day 2, prior to the addition of cyclopamine to the medium for dorsalization, I transiently expressed two pro-neurogenic pallial transcription factors, Ngn2 and NeuroD4. During neocortical development, Ngn2 is critical for induction of neurogenesis (Fode *et al.*, 2000; Schuurmans *et al.*, 2004). NeuroD4 is a pallial-restricted Ngn2 transcriptional target and cofactor that accelerates transcription of Ngn2 target genes (Mattar *et al.*, 2008).

During the remainder of this protocol of directed differentiation, I sequentially expressed, at two-day intervals, sets of transcription factors corresponding to progenitor, immature post-mitotic, and mature post-mitotic stages of CFuPN development. The first set of transcription factors (Sox11, Sox4, and Fezf2), expressed on day 4, promotes CFuPN progenitor specification. Fezf2 is required for the specification of sub-cerebral CFuPN (Molyneaux *et al.*, 2005; Chen *et al.*, 2005). Sox11 and Sox4 are required for neocortex-specific expression of Fezf2 (Shim *et al.*, 2012). The second set replicates immature post-mitotic CFuPN differentiation with co-expression of Ctip2, Tbr1, and Sox5; these transcription factors regulate the differentiation of distinct CFuPN subtypes. Subcerebral projection neurons (SCPN) express Fezf2 and Ctip2 more highly than Tbr1 and Sox5. Conversely, corticothalamic projection neurons (CThPN) express Tbr1 and Sox5 more highly; both of these transcription factors repress Fezf2 and SCPN identity (Tbr1, Bedogni *et al.*, 2010; McKenna *et al.*, 2011; Sox5, Lai *et al.*, 2008; Shim *et al.*, 2012). Although these transcription factors are co-expressed at early developmental time points, their expression refines to distinct subtypes during late corticogenesis (Azim *et al.*, 2009; Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). Our final, third set of transcription factors biases toward SCPN subtype specification by reinforcing the sustained expression of Fezf2 and Ctip2, with the additional expression of Otx1, which directs pruning of SCPN axonal projections (Weimann *et al.*, 1999).

At the peak expression of Nestin-expressing neural progenitors, on day 14, these modRNA-treated, ES-derived cells were assessed for neuronal induction on the basis of Tau::GFP expression (**Figure 4.1**). ES-derived cells sequentially transfected with modRNA coding for all transcription factors ("12-mod") show a large enrichment of Tau-expressing neurons relative to the untreated cells.

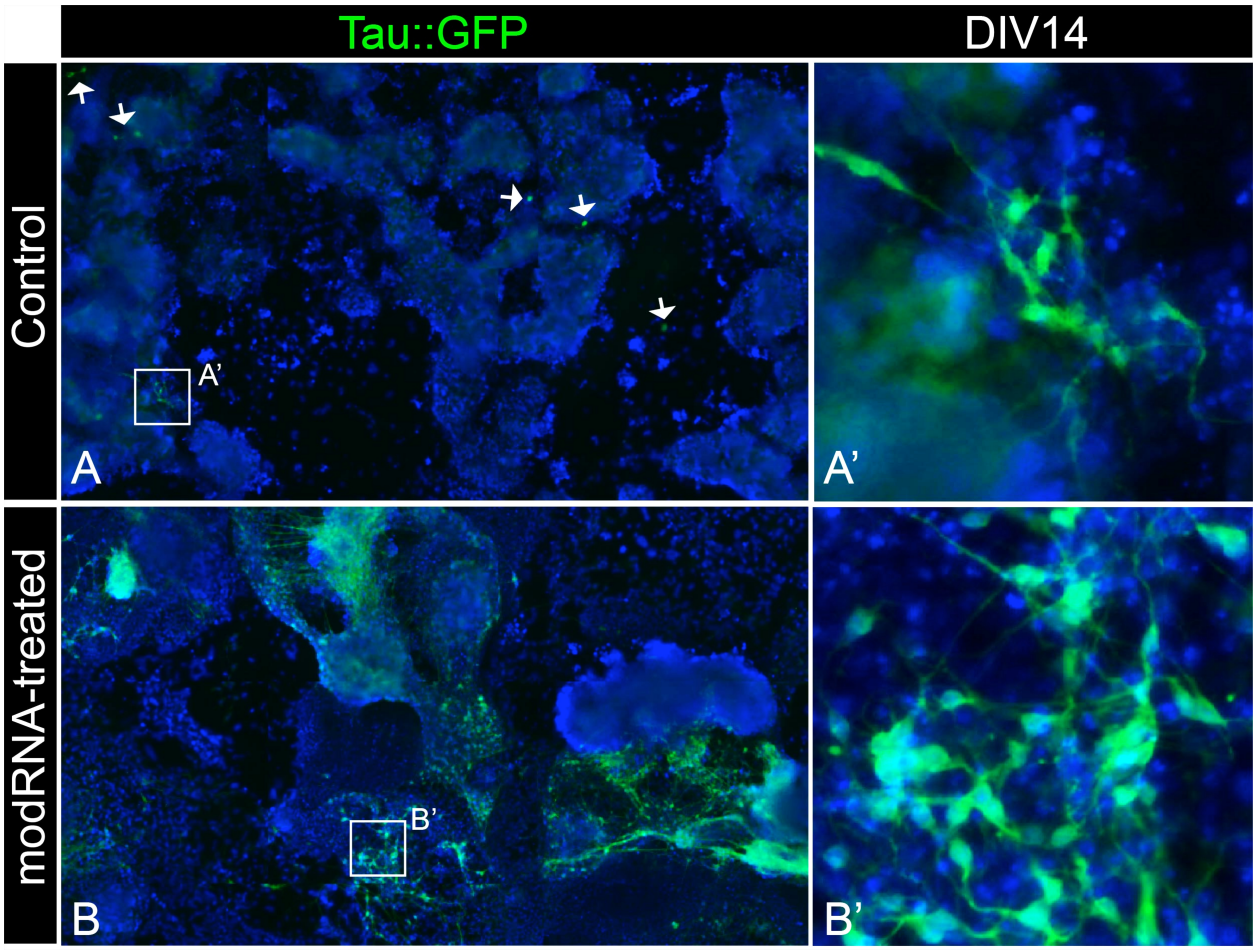

Figure 4.1. Sequential and transient induction of twelve transcription factors using modRNA enriches proportion of Tau-expressing, ES-derived neurons. (A) Tau::GFP mouse ES cells were differentiated for 14 days using an established monolayer protocol that generates pallial-like progenitors (Gaspard *et al.*, 2009). (B) Following sequential transfections, totaling twelve modRNAs coding for forebrain, neocortex, and CFuPN transcription factors (**Table 4.1**), Tau::GFP mouse ES cells generate enriched numbers of GFP-expressing neurons (N = 4).

The complete sequence of twelve transcription factors optimally improves directed generation of ES-derived corticofugal neurons

To further isolate which factors were essential for neuronal enrichment in our modRNA-directed differentiation protocol, I selectively removed individual transcription factors and assessed the impact on the generation of neurons (**data not shown**). I find that early pro-neural factors (especially Sox2, Ngn2, NeuroD4) are essential for a substantial fraction of the neuronal enrichment. Strikingly, removing the expression of late sets of transcription factors also diminished the neuronal enrichment, suggesting that the overall sequence could not be simplified to a smaller set of factors without compromising the quality of directed differentiation.

Given that each transfection step does not target all cells, I asked whether neuronal enrichment might be improved with the fully penetrant delivery of all factors in all cells. To test this hypothesis, I devised an alternate positive-selection protocol to enrich for cells that received all twelve transcription factors. I first cloned puromycin resistance (PuroR) modRNA. Next, I assessed whether PuroR acutely and transiently protects transfected ES-derived cells during the application of puromycin antibtiotic (**Figure 4.2**). I find that PuroR lasts approximately 48hrs, consistent with the kinetics of most modRNA-expressed genes. These kinetics suggest that PuroR modRNA can be effectively used for positive selection of transfected cells. Addition of PuroR modRNA at each stage of the modRNA directed differentiation protocol would ensure that only cells that have received each set of transcription factors during progressive rounds of modRNA transfection will survive selection with puromycin. However, we find that this approach generates neurons at a similar density as we had previously observed, and could not be distinguished from neurons undergoing the original protocol (**data not shown**).

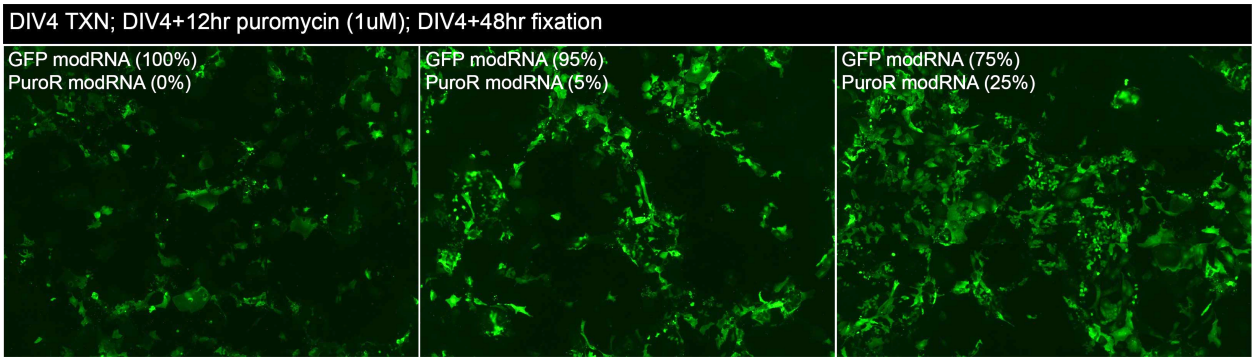

Figure 4.2. Transient expression of puromycin resistance gene acutely selects for transfected ES-derived cells following puromycin treatment. Wild-type mouse ES cells were differentiated using an established monolayer protocol that generates pallial-like progenitors (Gaspard *et al.*, 2009). On the fourth day of differentiation, these cells were transfected with modRNA encoding GFP and/or Puromycin resistance (PuroR). Twelve hours following transfection, puromycin antibiotic was added to the medium. On the sixth day of differentiation, cells were fixed and immunolabeled for GFP. Panel on the left demonstrates puromycin-mediated cell death and loss of viable GFP-transfected cells, in the absence of PuroR. Panel on the right shows viability and puromycin-mediated selection of GFP / PuroR co-expressing cells following PuroR co-transfection.

Micro-transplanted ES-derived project axons specifically to corticofugal targets

To assess whether "12-mod" treated ES-derived neurons have acquired corticofugal projection neuron class identity, I directly micro-transplanted the heterogeneous mix of ES-derived neurons and other cells obtained at the end of our modRNA directed differentiation protocol into early postnatal mouse neocortex. These micro-transplantation experiments differ from previously published studies in two important respects. First, I micro-transplanted only 2-3,000 cells in a total volume of ~50nL. In contrast, other groups injected a 100-fold larger number of ES-derived cells (Gaspard *et al.*, 2008; Espuny-Camacho *et al.*, 2013), which potentially obscures cell autonomous projection decisions by causing clumping and sequestration from the *in vivo* microenvironment. Because of the high enrichment of neurons in our differentiation protocol (**Figure 4.2**), there are enough neurons to observe axon outgrowth without FACS enrichment.

Second, the depth of a single stereotactic injection was limited to the neocortical parenchyma. Avoiding the subcortical white matter and striatum enables transplanted neurons to project according to their intrinsic identity, rather than fasciculating with and following host projections, as occurs when embryonic neocortical cells are transplanted into the deep white matter (Fricker-Gates *et al.*, 2002; Wuttke and Macklis, unpublished data, 2013). If transplanted more deeply into the dorsal striatum, the axonal projections of transplanted neurons become even more distributed, following multiple non-specific projection patterns (Magavi and Lois, 2008). I defined strict *a priori* exclusion criteria, rejecting samples containing injections in the white matter (or deeper) from further analysis.

I first assessed non-modRNA-treated ES-derived neocortical cells as a control, and find that there are no projections in the pyramidal tract, though a few stray axons were found in the

midbrain (**Figure 4.3**). The differences between my current results and the original report showing specific visual/limbic axonal projections from large transplants of heterogeneous, ES-derived neocortical-like neurons (Gaspard *et al.*, 2008) include: 1) my protocol of transplantation avoids non-cell autonomous axon fasciculation; 2) my analyses were done within one week of micro-transplantation, rather than after 28 days in alternate protocols, which could have incorporated the continued neurogenesis of Tau::GFP-negative ES-derived progenitors or allowed potential axonal pruning.

I next assessed the transplantation of "12-mod"-treated ES-derived neurons. Within three days of micro-transplantation into the postnatal neocortex, these modRNA-directed ES-derived Tau::GFP neurons send numerous projections specifically toward the internal capsule, while axons in the corpus callosum are rare (**Figure 4.4**). By six days post-transplantation, the axonal processes of ES-derived transplanted neurons have continued along the pyramidal tract, reaching the midbrain (**Figure 4.5**). Strikingly few axon terminals are present in contralateral cortex.

Figure 4.3. Untreated, ES-derived neurons micro-transplanted in post-natal neocortical parenchyma do not project their axons along the pyramidal tract. Tau::GFP mouse ES cells were differentiated for 14 days using an established monolayer protocol that generates pallial-like progenitors and neocortical-like neurons (Gaspard *et al.*, 2009). Six days following micro-transplantation into P0 neocortical parenchyma, I assessed the axonal projections of transplanted, ES-derived neurons. (A) Rare, ES-derived, Tau::GFP axons are identified in the striatum at P6. (B) Rare, ES-derived, Tau::GFP axons in caudal sections are not located in the cerebral peduncle at P6 (N=2 littermates).

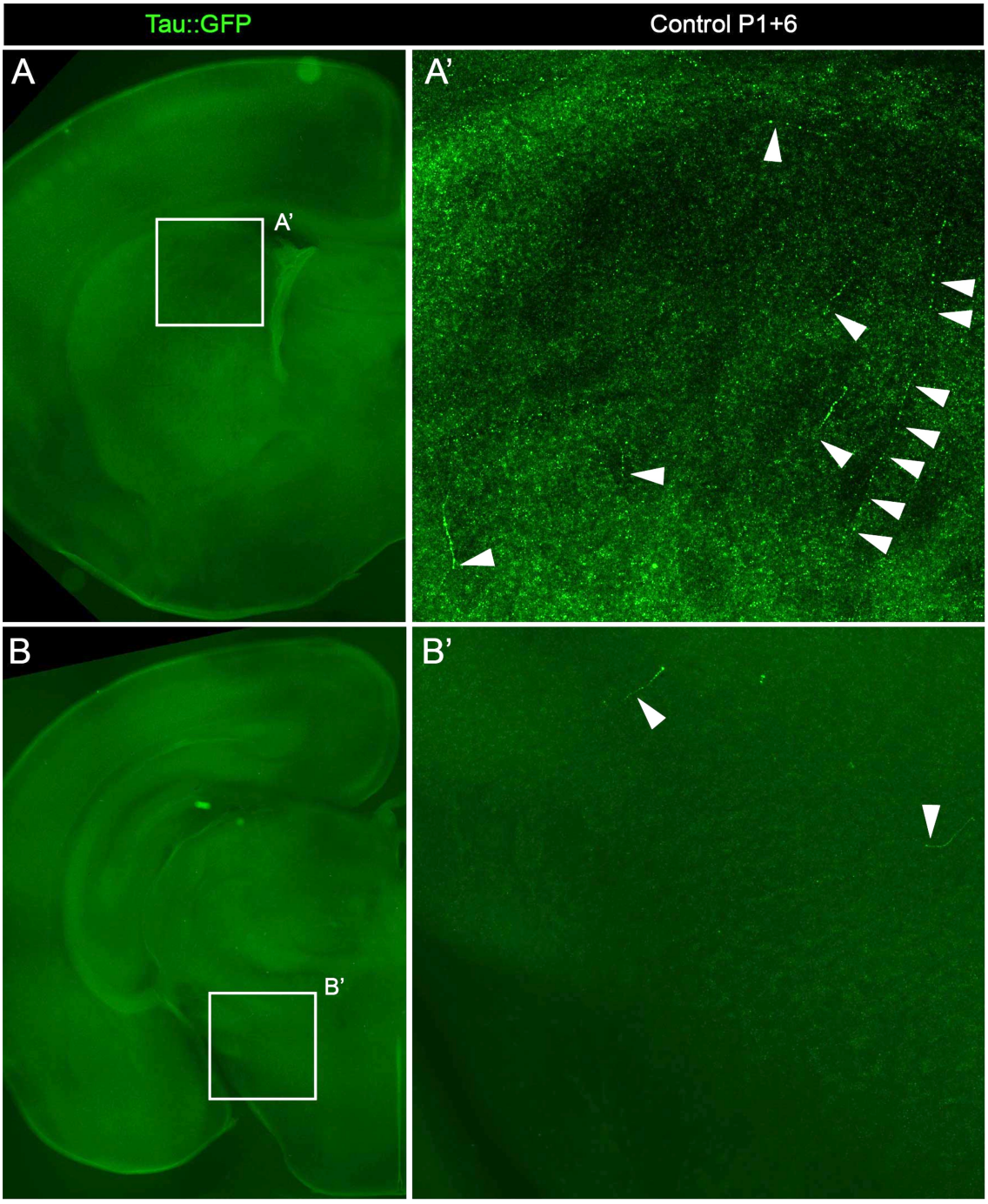

Figure 4.3 (Continued)

Figure 4.4. "12-mod"-treated, ES-derived neurons project their axons into the dorsal aspect of the internal capsule, three days following micro-transplantation in post-natal neocortical parenchyma. Tau::GFP mouse ES cells were sequentially transfected with a total of twelve distinct transcription factors (**Table 4.1**) over 14 days of differentiation, using an established monolayer protocol that generates pallial-like progenitors and neocortical-like neurons (Gaspard *et al.*, 2009). Three days following micro-transplantation into P0 neocortical parenchyma, I assessed the axonal projections of transplanted, ES-derived neurons. (A) Large numbers of ES-derived, Tau::GFP axons are identified in the striatum at P3. (B) The somata of ES-derived, Tau::GFP neurons are locally-dispersed and largely restricted to Ctip2-negative upper layers. (C) The majority of ES-derived, Tau::GFP axons are fasciculated and enter the internal capsule, whereas rare axons are found in the corpus callosum and contralateral neocortex. (N=2 littermates)

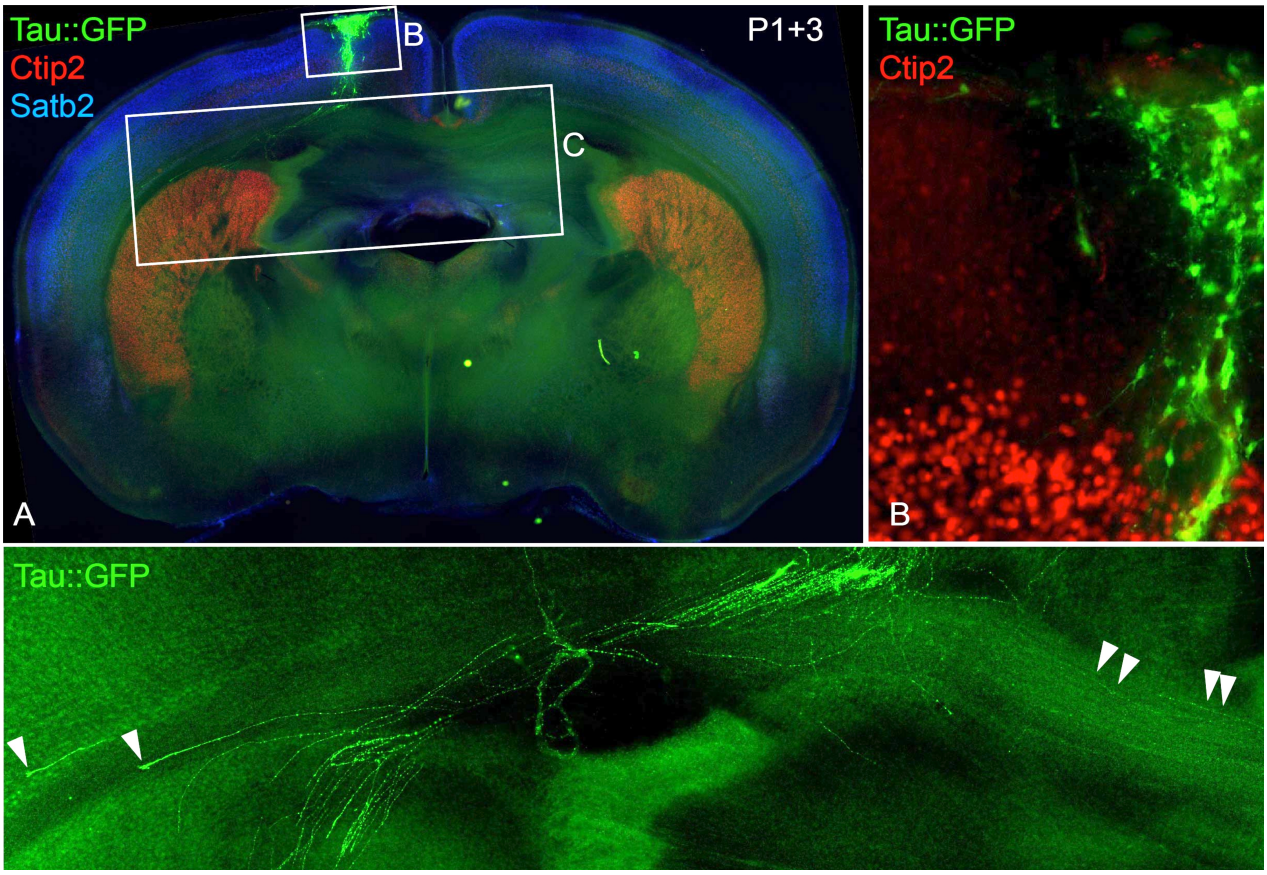

Figure 4.4 (Continued)

Figure 4.5. "12-mod"-treated, ES-derived neurons project their axons along the pyramidal tract and enter the cerebral peduncle, six days following micro-transplantation in post-natal neocortical parenchyma. Tau::GFP mouse ES cells were sequentially transfected with a total of twelve distinct transcription factors (**Table 4.1**) over 14 days of differentiation, using an established monolayer protocol that generates pallial-like progenitors and neocortical-like neurons (Gaspard *et al.*, 2009). Six days following micro-transplantation into P0 neocortical parenchyma, I assessed the axonal projections of transplanted, Tau::GFP ES-derived neurons. (A) Whole-mount immunofluorescence shows a single transplantation site in the neocortex. (B) Coronal section at the transplantation site reveals large numbers of ES-derived, Tau::GFP axons entering the internal capsule; only rare axons enter the contralateral neocortex. (C) 160um caudal to injection site, Tau::GFP axons are present in the dorsal internal capsule and avoid the contralateral cortex. (D) Caudal section shows Tau::GFP axons in the pyramidal tract, innervating thalamus. (E) Tau::GFP axons are localized within the pyramidal tract of the cerebral peduncle. (N=2 littermates)

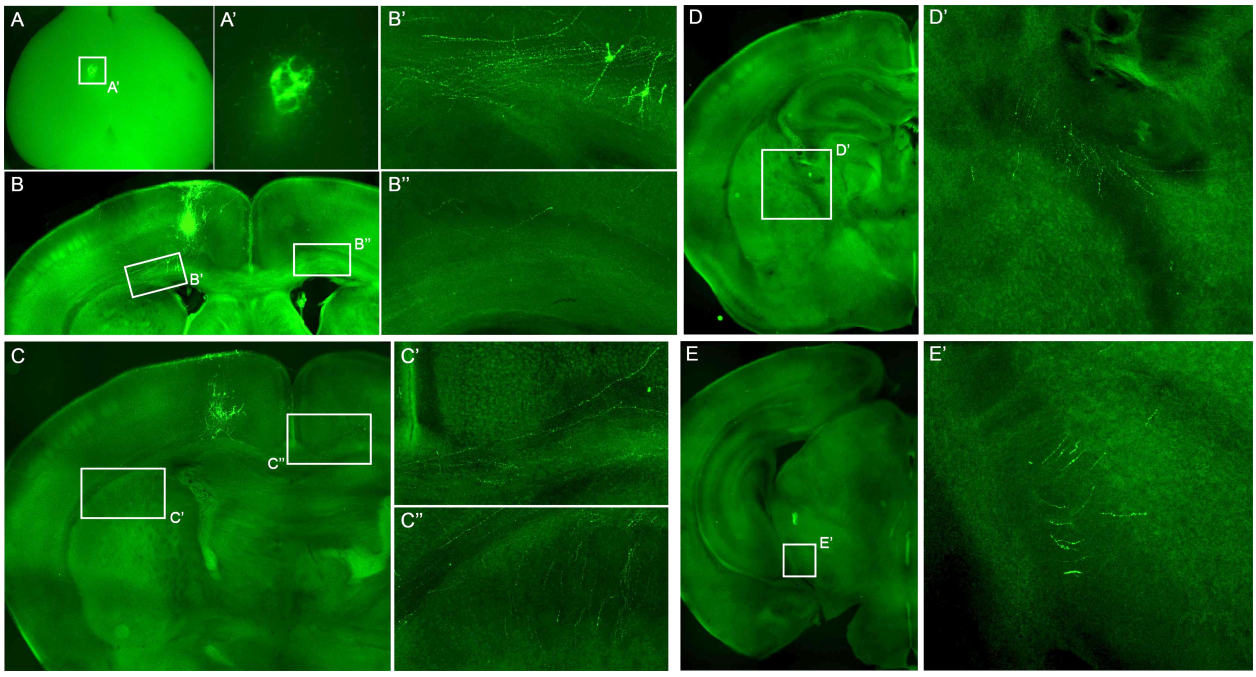

Figure 4.5 (Continued)

Micro-transplanted ES-derived neurons do not fuse with endogenous CFuPN

Early-stage ES-derived cells and ES cells themselves have been shown to fuse with primary neurons under specific conditions *in vitro* and *in vivo* (Ying *et al.*, 2002; Cusulin *et al.*, 2012). Similarly, fusion reportedly occurs between CNS-derived multi-potent neural progenitor cells (NPCs) *in vitro* (Chen *et al.*, 2006) and between NPCs and host neocortical neurons *in vivo* (Brilli *et al.*, 2012). In contrast, this phenomenon has not been observed after the transplantation of more mature, dissociated embryonic neocortical neurons into mouse neocortex or striatum (Fricker-Gates *et al.*, 2002; Gaillard *et al.*, 2007; Magavi and Lois, 2008; Wuttke and Macklis, unpublished data, 2013).

I investigated this theoretical possibility rigorously, and find that fusion is highly unlikely in my transplantation experiments, for the following reasons. First, the relative abundance of axons in the internal capsule compared to the corpus callosum; projection patterns would be expected to more closely resemble those of host neurons in the event of fusion, and two to three times as many host neurons project across the corpus callosum than in the internal capsule (**Figure 4.4, Figure 4.5**). Second, differentiation of ES-derived neurons progressively unfolds over the course of six days; if fusion had occurred, gradual growth of axons along the pyramidal tract would not have been observed. Third, the position of micro-transplanted neurons was mostly in the upper layers, so the bias toward corticofugal neurons cannot be explained by fusion with neurons at the transplantation location. Fourth, expression of Ctip2 is detectable only in rare cells; many more ES-derived neurons would be expected to be Ctip2-positive if fusion had occurred (**Figure 4.6**).

To more rigorously exclude the possibility of cell fusion, I conducted a two-color transplantation experiment in which Tau::GFP ES-derived neurons were transplanted into a Rosa26::tdTomato mouse. I find that none of the transplanted neurons acquire expression of

tdTomato (**data not shown**). While these experiments do not completely rule out the possibility of rare fusion events, such low frequency events could not account for the corticofugal projections from ES-derived neurons.

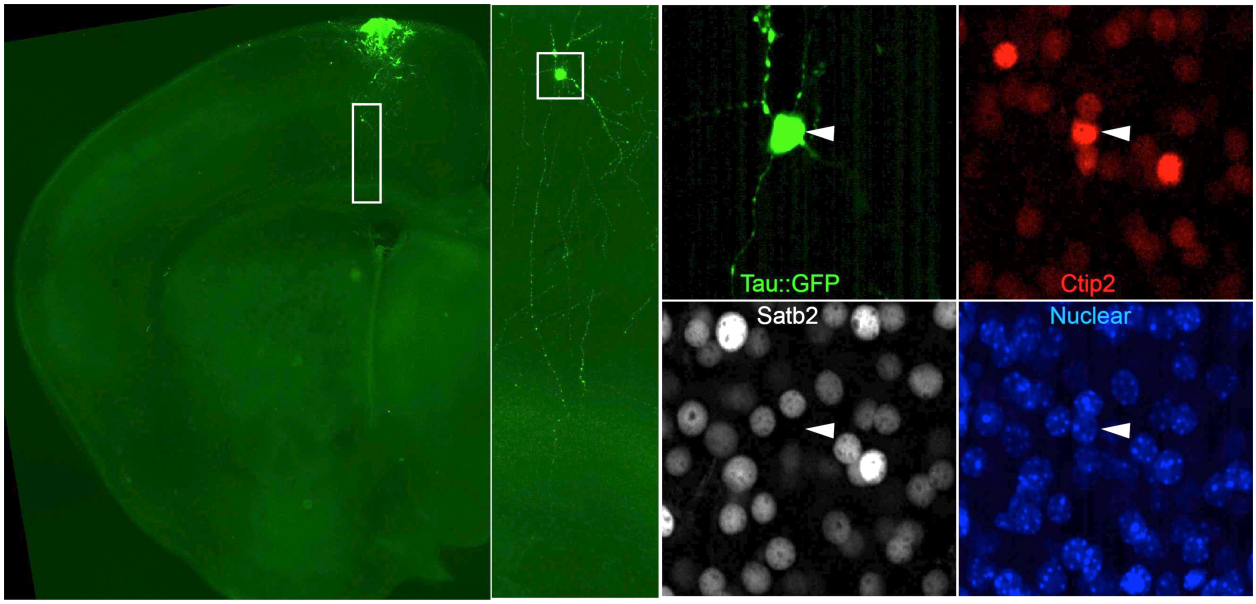

Figure 4.6. Ctip2 is not expressed by most "12-mod"-treated, ES-derived neurons, six days following micro-transplantation in post-natal neocortical parenchyma. Tau::GFP mouse ES cells were sequentially transfected with a total of twelve distinct transcription factors (**Table 4.1**) over 14 days of differentiation, using an established monolayer protocol that generates pallial-like progenitors and neocortical-like neurons (Gaspard *et al.*, 2009). Six days following micro-transplantation into P0 neocortical parenchyma, I assessed the expression of Ctip2 and Satb2 by transplanted, ES-derived neurons, and show one Tau-expressing neuron with a corticofugal axon that co-expresses Ctip2 and does not co-express Satb2; all other neurons were negative for both Ctip2 and Satb2 (N = 25 isolated intraparenchymal neurons).

4.4 Discussion

I have assessed whether the sequential and transient expression of multiple transcriptional regulators over CFuPN development can direct the differentiation of mouse ES cells into CFuPN. My results show that this approach accelerates neurogenesis in a previously established monolayer protocol of heterogeneous, neocortical-like differentiation (Gaspard *et al.*, 2008; **Chapter 2**; **Figure 4.2**). When micro-transplanted into neocortical parenchyma, a significant proportion of these modRNA-treated neurons are CFuPN-like, specifically directing their axonal projections to distinct targets in the thalamus, striatum, and pyramidal tract consistent with at least two types of CFuPN: corticothalamic and subcerebral projection neurons (**Figure 4.5**).

My findings indicate the first substantial enrichment of a single broad neocortical class (CFuPN) derived from mouse ES cells, as assessed by micro-transplantation. Refinements of this initial approach might enable increasingly specific directed differentiation of other classes, types, and subtypes of neocortical neurons. In contrast, prior reports using the monolayer protocols of morphogen-based pallial-like progenitor specification have generated heterogeneous populations expressing characteristics of neocortical-like neurons (Gaspard *et al.*, 2008; Mariani *et al.*, 2012; Shi *et al.*, 2012; Espuny-Camacho *et al.*, 2013; **Chapter 2**).

Previous efforts to generate CFuPN have focused on the transcription factor Fezf2, a "master" regulator of sub-cortical CFuPN (SCPN) specification (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Shim *et al.*, 2012). However, increasing evidence suggests that Fezf2 requires a specific molecular context to fully activate CFuPN molecular identity (including, but not limited to, Ctip2 expression) and to fully repress CPN molecular identity (*e.g.*, Satb2), both *in vivo* (Chen *et al.*, 2008; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013) and *in vitro* (**Chapter 3**; Wang *et al.*, 2011). I have previously shown that the molecular identity

of ES-derived neocortical-like neurons is incompletely specified, resulting in stalled neocortical maturation (**Chapter 2**). I have also shown that Fezf2 expression, when preceded by CFuPN-specific epigenetic priming (Sirt1 inhibition), helps refine CFuPN molecular identity, but cannot enrich its quantity (**Chapter 3**).

Because deficits in early neocortical differentiation are observed using previously published morphogen-based protocols (**Chapter 2**), my novel modRNA-based approach begins at the earliest stages of ES cell differentiation and neural induction, in order to promote more uniform progenitor differentiation. The major molecular pathways chosen for this approach recapitulate a schematized progression through major milestones of CFuPN development (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012) (**Table 4.1**).

Synthetic modified mRNA (modRNA) technology enables greater precision over transient gene dosing than is available with similar methods such as transfection (Warren *et al.*, 2010; **Chapter 2**). In contrast to alternate methods of gene expression, this modRNA-based approach has several advantages. First, transient expression enables the expression of a temporally restricted sequence of transcription factors normally active during neural tube differentiation. Second, the co-expression of multiple modRNAs permits stoichiometric gene dosing, which can be used to further optimize this protocol in the future. Third, the kinetics of modRNA expression and degradation are consistent with near-physiological ranges and oscillations of gene dosing. For example, multiple regulators of neocortical differentiation are expressed in a cyclical or cell cycle-dependent manner (*e.g.*, Notch/Hes, Ngn2, and Nestin; Kageyama *et al.*, 2008; Sunabori *et al.*, 2008). Additionally, several transcription factor controls are known to regulate differentiation in a dose-dependent manner (*e.g.*, Pax6, Sox2, and Otx1/2; Schedl *et al.*, 1996; Manuel *et al.*, 2007; Georgala *et al.*, 2011; Kopp *et al.*, 2008; Acampora *et al.*, 1999). Fezf2, Sox5, Ctip2, and Tbr1 are likely also dose-sensitive during

neocortical development, on the basis of expression analysis (Molyneaux *et al.*, 2005; Lai *et al.*, 2008; Arlotta *et al.*, 2005; Bedogni *et al.*, 2010; McKenna *et al.*, 2011), and on the basis of observed phenotypic abnormalities in heterozygous mice, particularly for Ctip2 and Fezf2 (Arlotta *et al.*, 2005; Galazo and Macklis, unpublished data, 2013). Finally, for reasons that are still unclear, in comparison to virus-mediated reprogramming, modRNA more quickly and efficiently induces reprogramming (Warren *et al.*, 2010); I speculate that this might result from near-physiologic gene dosing.

Moreover, transient gene dosing might promote stable and high fidelity subtype identity acquisition. Neurons that are already committed to alternate fates are unlikely to be reprogrammed by transient gene expression; multiple lines of evidence suggest that above-physiological transgene levels are required to override a stable transcriptional network (Hochedlinger and Plath, 2009; Ladewig *et al.*, 2013). The constitutive misexpression of normally transiently expressed transcription factors frequently generates hybrid cell types, owing to residual epigenetic marks from the original cell type (reviewed in Vierbuchen and Wernig, 2012, 2011). For example, hepatocytes converted to neuron-like cells by constitutive expression of Mash1, Brn2, and Myt1l maintain residual expression of hepatocyte-specific genes (Marro *et al.*, 2011); fibroblasts converted to dopaminergic neuron-like cells, by constitutive expression of Mash1, Nurr1, and Lmx1a also maintain numerous transcriptional differences when compared to authentic midbrain dopaminergic neurons (Caiazzo *et al.*, 2011). I speculate that ES cells at a transitional developmental stage might be most appropriately biased toward an endogenously stabilized neuronal identity with transient gene dosing.

This modRNA approach does not target all cells; since the penetrance of transfection is approximately 40-50% (lower than the reported 80-90% within fibroblasts; Warren *et al.*, 2010), most cells receive an incomplete complement of factors (**Figure 4.1**). This likely explains why

all factors appear to contribute to the enrichment of neurons (**Figure 4.3**). However, the incomplete expression of factors likely does not inhibit the enrichment of neurons. When ES-derived cells that receive all factors are selected by sequential puromycin dosing (**Figure 4.4**), the proportion of Tau-expression is comparable to the unselected condition (**Figure 4.4**).

In addition to this novel strategy of directed differentiation, I utilize micro-transplantation as a highly rigorous approach for assessment of ES-derived neocortical axonal projection subtype identity (adapted from (Wuttke and Macklis, unpublished data, 2013). In contrast to prior protocols of transplantation into postnatal animals that examined the transplantation of many cells into the neocortical white matter, I injected very small numbers of neurons into relatively superficial neocortical parenchyma. I made these protocol changes because large clumps of cells can mask neuronal identity by enabling non-specific axonal projections along nearby white matter tracts (Gaspard *et al.*, 2008; Espuny-Camacho *et al.*, 2013). In addition, even small numbers of transplanted cells placed in the deeper subcortical white matter and the striatum result in exuberant, non-specific projections within the white matter (Magavi and Lois, 2008). This micro-transplantation approach provides substantially refined phenotypic and functional analysis of ES-derived neurons.

Fusion does not account for the neuronal projection patterns of ES-derived transplanted neurons. First, micro-transplanted neurons robustly projected axons only as far as the dorsal aspect of the internal capsule after 3 days, and the number of axons entering the midbrain at 6 days was similar. This growth pattern is most consistent with transplant-derived axon extension. Second, GFP-positive axons in the corticofugal tract were more abundant, relative to innervation of contralateral cortex. Given that contralateral cortical innervation is significantly more abundant by native cortical neurons, these data are most consistent with transplant-derived axons preferentially projecting to corticofugal targets. Third, the position of

micro-transplanted neurons was mostly in the upper layers, so the bias toward corticofugal neurons cannot be explained by fusion with neurons at the transplantation location. Fourth, when transplanting Tau::GFP neurons into a Rosa26::tdTomato mouse, no transplanted neurons expressed tdTomato; this does not exclude rare fusion events, but such events cannot account for the observed axonal projections.

The absence of Ctip2 expression in micro-transplanted ES-derived neurons is striking (**Figure 4.6**; and data not shown). This expression profile is inconsistent with normal CFuPN development. However, there is a substantial precedent for Ctip2-deficient CFuPN-directed reprogramming (Chen *et al.*, 2008; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013). Given simplified media and differentiation conditions, I speculate that ES-derived CFuPN might harbor multiple molecular deficits. It remains to be explored what other molecular distinctions exist between ES-derived CFuPN and the corresponding types and subtypes of CFuPN *in vivo*.

Overall, these findings indicate that the delineation of distinct transcriptional cascades in CFuPN development permits a rational approach to directed differentiation of mouse ES-derived neurons to CFuPN. Sequentially expressing transient developmental programs might be essential for the optimal specification of neuronal subtypes that continue to post-mitotically refine their identity over an extended period, which is theoretically necessary for precise neocortical development. By providing critical transcriptional cues, and allowing endogenous responses to execute cell fate specification, this modRNA-based approach helps ensure the proper development of ES-derived CFuPN. Further refinements to this protocol (*e.g.*, epigenetic priming, **Chapter 3**; or modified dosing or composition of specific transcription factors) might enable the specification of more precisely defined projection neuron subtypes, including the clinically important population of corticospinal motor neurons. This modRNA-based approach to directed neocortical subtype differentiation might be easily and safely

applied to directed differentiation of human pluripotent cell types.

4.5 Experimental Procedures

Cell culture and differentiation

Murine embryonic stem cells: Feeder-free E14Tg2a (Baygenomics) mouse ES cells were propagated using standard procedures (Ying *et al.*, 2003) on gelatin-coated (0.1% gelatin, StemCell Technologies) cell culture treated plastic dishes. Tau-GFP knock-in ES cells (gift of Kerry Tucker, University of Heidelberg) were routinely propagated on mitotically inactivated mouse embryonic fibroblasts (Millipore) on gelatin-coated cell culture treated plastic dishes.

Mouse embryonic stem cell media is GMEM (Invitrogen) supplemented with 10% ESC-certified fetal bovine serum (vol/vol, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM β-mercaptoethanol (Sigma), 50 U/mL penicillin/streptomycin and 1000 U/mL leukemia inhibitor factor (ESGRO).

For differentiation, ES cells were plated at low density (5,000 cells/cm^2) on gelatin-coated plastic dishes in ES cell medium, and cultured as described (Gaspard *et al.*, 2009). Briefly, ESCs were trypsinized, dissociated, and plated on gelatin-coated cell culture plates. Medium was changed to DDM after one day.

DDM consists of DMEM/F12 (Invitrogen-Gibco) supplemented with N2 supplement (N2 supplement consists of 8.61uM insulin, 1mM transferrin, 2uM progesterone, 10mM putrescine and 3uM selenite; Invitrogen-Gibco), 2mM glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 0.5 mg/ml bovine serum albumin fraction V (all from Invitrogen-Gibco), and 0.1mM β-mercaptoethanol (Sigma).

Cyclopamine (Calbiochem) was added from day 2 to day 10 in the differentiation medium at a final concentration of 1uM. After 10 to 14 days of differentiation, cells were

trypsinized, dissociated and plated on poly-lysine/laminin (Becton-Dickinson) coated glass coverslips and allowed to grow for 4–14 days in N2B27 medium.

N2B27 medium consists of a 1:1 mixture of DDM and Neurobasal that is supplemented with B27 (without vitamin A; Invitrogen-Gibco) and 2 mM glutamine.

Visualization and Analysis

Wide-field image acquisition was performed using a Nikon 90i epifluorescence microscope with a Clara DR-328G cooled CCD digital camera (Andor Technology, Belfast, Northern Ireland). Confocal imaging was performed with a BioRad Radiance 2100 Rainbow laser-scanning confocal microscope based on a Nikon E800 microscope. Images were assembled in Adobe Photoshop and Illustrator (CS3, CS5), with adjustments for contrast, brightness, and color balance to obtain optimal visual reproduction of data.

RNA synthesis and transfection

modRNA was generated as per (Warren *et al.*, 2010). Briefly, RNA was synthesized with the MEGAscript T7 kit (Ambion, Austin, TX). A custom ribonucleoside blend was used comprising 6 mM 5’ cap analog (New England Biolabs), 7.5 mM adenosine triphosphate and 1.5 mM guanosine triphosphate (USB, Cleveland, OH), 7.5 mM 5-methylcytidine triphosphate and 7.5 mM pseudo-uridine triphosphate (TriLink Biotechnologies, San Diego, CA). Reactions were incubated 3–6 hr at 37C and DNase treated as directed by the manufacturer. RNA was purified with Ambion MEGAclear spin columns, then treated with Antarctic Phosphatase (New England Biolabs) for 30 min at 37C to remove residual 5’ triphosphates. Treated RNA was repurified, quantitated by Nano-drop (Thermo), and adjusted to 100ng/mL working

concentration by addition of Tris-EDTA (pH 7.0). modRNA transfections were carried out with RNAiMAX (Invitrogen), as per manufacturer's instructions. To minimize the toxicity of repeat transfection, the lipofectamine-containing media was replaced with DDM after 4-5hrs.

Mice

All mice used in these experiments were handled according to guidelines of the National Institutes of Health (NIH), and all procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of Harvard University. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Rosa26::tdTomato mice were acquired from Jackson Laboratories. Wild-type CD1 mice were used in all other experiments (Charles River Laboratories).

Transplantation into early postnatal neocortex

ES-derived cells were concentrated to a density of approximately 100 million cells per mL. P0-P1 mice were deeply anesthetized on ice for 3-4 minutes. A small incision in the skull was made with a needle. Cells were delivered using a digitally controlled nanoinjection system (Nanoject Variable, Drummond) via a glass micropipette with an outer diameter of 80-100 μm. Approximately 5-6 single injections of 4.6nL volume (total: 20-30nL; ~2,000 cells) were placed at multiple depths in a single tract, no deeper than 100um below the pial surface, positioned 1mm lateral and 1mm rostral to bregma. Only sample brains that did not have white matter injections were included in the analyses.

Immunocytochemistry

Mice were deeply anesthetized with a lethal dose of anesthetic (Avertin) or hypothermia, and perfused trans-cardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde, rinsed with PBS, and sectioned coronally with 50μm thickness on a vibrating microtome (Leica). Sections were blocked in 1% BSA (Sigma), and 0.3% Triton X-100 (Sigma) for 1hr at room temperature before incubation with primary antibodies overnight at 4°C. Cells were fixed in 4% paraformaldehyde (wt/vol) for 30 min and washed three times in phosphate-buffered saline (PBS) before incubation with blocking reagent and primary antibodies for 1hr. Primary antibodies and dilutions used were: rat antibody to Ctip2 (1:500, Abcam), mouse antibody to Satb2 (1:200, Abcam), rabbit antibody to Ctip1 (1:500, Abcam), rabbit antibody to GFP (1:500, Invitrogen), rabbit antibody to Ki67 (1:500, Abcam), chicken antibody to Nestin (1:500, Novus Biologicals), mouse antibody to TuJ (1:500, Covance), mouse antibody to Map2 (1:500, Sigma), and mouse antibody to NeuN (1:250, Millipore). Alexa fluorophore conjugated secondary antibodies from Invitrogen were used at a dilution of 1:1000. Hoechst 33342 counterstain was used to visualize nuclei (1:3,000, Invitrogen).

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Chapter 5

Discussion

5.1 Overview

During neocortical development, diverse projection neuron classes, types, and subtypes are generated under temporal and spatial regulation of cell-extrinsic and cell-intrinsic molecular controls (Molyneaux *et al.*, 2007; Fame *et al.*, 2011; Woodworth *et al.*, 2012; MacDonald *et al.*, in press; Custo Greig *et al.*, in editorial revision, 2013). Neocortical projection neurons can be broadly classified into two groups: corticofugal projection neurons (CFuPN) and commissural projection neurons, which primarily include callosal projection neurons (CPN). CFuPN are the primary output neuron population of the neocortex, and their axons innervate sub-cortical targets including thalamus, striatum, brainstem, and spinal cord. In contrast, the axons of CPN comprise the corpus callosum, which bridges the two cerebral hemispheres. Together, CFuPN and CPN account for the vast majority of neocortical projection neurons; included within each of these distinct classes of neurons are potentially hundreds of neuronal subtypes intermixed in distinct neocortical layers and areas, with distinct axonal targets.

In this dissertation, I introduce multiple approaches that apply an emerging understanding of neocortical development both to evaluate and direct mouse embryonic stem (ES) cell differentiation to CFuPN, the primary output neurons of the neocortex. Although ES cells are theoretically competent to generate CFuPN *in vitro*, existing ES cell differentiation protocols are not directed toward specific classes, types, or subtypes of neocortical projection neurons and instead rely on the spontaneous generation of neurons with neocortical-like characteristics. In Chapter 2, after successfully replicating these established protocols, I present experimental results indicating that these spontaneously-generated ES-derived neocortical-like neurons are heterogeneous, immature, and “stalled” at a stage roughly equivalent to mid-embryonic differentiation *in vivo*. In Chapter 3, I build on this foundation to promote CFuPN-specific differentiation among these “stalled” neocortical-like neurons, using a

combination of recently developed synthetic modified mRNA (modRNA) and high-content chemical screening technologies. Finally, in Chapter 4, I direct differentiation of ES cells to CFuPN by sequentially and transiently expressing critical forebrain, neocortex, and CFuPN-specific transcription factors. When micro-transplanted into neonatal neocortex, these ES-derived neurons appropriately innervate corticofugal targets in the thalamus and midbrain. Overall, using these biological and technical approaches, I rigorously characterize and then successfully enhance the directed differentiation of ES cells into CFuPN.

In this final chapter of my dissertation, I critically review my experimental findings and discuss topics of directed differentiation that have broad relevance to the field, including: 1) commonalities of maturation defects in many *in vitro* protocols of directed differentiation, potentially owing to "confusion" of molecular identity; 2) potential mechanisms causing immature or "confused" *in vitro* differentiation; and 3) strategies for directed differentiation of clinically important subtypes (*e.g.* CSMN) within the broad class of CFuPN. Together, these topics delineate the broader challenges and long-term applications of my work for *in vitro* neurodegenerative disease modeling, particularly toward developing new treatments for amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI).

5.2. Maturation deficits in protocols of ES cell directed differentiation

Neocortical projection neuron classes, types, and subtypes undergo distinct molecular refinements under the sequential control of transcriptional regulators at progenitor and post-mitotic stages of development (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013). In the absence of transcriptional regulators that control this refinement, precise molecular identity, laminar/areal positioning, and/or projection patterns of neocortical projection neuron subtypes are disrupted *in vivo*. Critical transcriptional controls, therefore, are promising candidates for both rigorous characterization of *in vitro* ES-derived neocortical-like neurons and for CFuPN-directed differentiation. Recent advances in the largely spontaneous differentiation of ES cells recapitulate some, but not all, aspects of corticogenesis. These protocols are largely based on the sequential expression of single genes characteristic of neocortical neurons (Hansen *et al.*, 2011).

My experiments in Chapter 2 and Chapter 3 are the first to systematically assess multiple stage-specific characteristics of neocortical differentiation *in vitro*, and my findings identify distinct deficits of these ES-derived pallial-like progenitors and neocortical-like neurons. These conclusions are based on the results of four inter-related experiments. First, ES-derived pallial-like progenitors are more heterogeneous than their *in vivo* counterparts and seemingly include many incorrectly specified progenitors (*e.g.*, heterogeneous expression of pallial and forebrain transcriptional regulators Pax6, Sox6, and Otx2; **Figure 2.2**). Second, ES-derived neurons are not uniformly or completely mature, but some display crucial hallmarks of early maturation (*e.g.*, TuJ1, Map2, NeuN), roughly equivalent to neocortical neurons *in vivo* at E16.5-E18.5 (**Figure 2.4**). Third, these immature neocortical-like neurons co-express multiple critical transcription regulators (*e.g.*, Tbr1, Ctip2, Satb2, Ctip1), consistent with the broad co-

expression of these genes by immature neocortical projection neurons at an equivalent *in vivo* stage of mid-corticogenesis (**Figure 2.3; Figure 2.5**). Fourth, these "stalled" neocortical-like neurons appropriately co-express some, but not all, post-mitotic controls over area-specific differentiation (*e.g.*, CoupTF1, Bhlhb5, Ctip1), which potentially indicates deficits in area-specific neocortical differentiation (**Figure 2.6**). My findings support the conclusion that ES-derived neocortical-like neurons generated by an established monolayer protocol most closely resemble *in vivo* neocortical neurons stalled at mid-corticogenesis and display features of incomplete neocortical specification.

One of the more striking implications of incomplete neocortical neuronal specification is that the molecular context of ES-derived neocortical-like neurons is not sufficiently permissive for SCPN specification by transient modRNA-based Fezf2 expression (**Figure 3.3**). In contrast, the molecular context of *in vivo* pallial progenitors is sufficiently permissive for SCPN specification by transient modRNA-based expression of Fezf2 at E12.5 (**Figure 3.1E-I**). These data presented in my dissertation (**Chapter 3**) and in the existing literature (Wang *et al.*, 2011) strongly indicate that a sufficiently permissive molecular context of differentiation is critical to enable Fezf2-directed CFuPN differentiation *in vitro* and further highlight the insufficient specification and maturation of these populations of heterogeneous ES-derived neocortical-like neurons.

Specification and maturation defects are broadly reported in the field of directed differentiation

Deficits in the differentiation ES cells to mature neuronal populations are commonly observed in the broader field of directed differentiation. These maturation deficits are hypothetically due to simplified media conditions and to a potentially common biology of neuronal specification. For example, neocortical projection neurons are not the only neuronal

population that displays increasingly restricted expression of subtype-specific transcription factors during maturation *in vivo*; indeed, spinal motor neurons (SMN) follow a similar process of refinement and diversity generation (Jessell, 2000; Dasen and Jessell, 2009; Alaynick *et al.*, 2011). Initially, early post-mitotic SMN express the transcription factors Hb9, Islet1, and Lhx3 (Sharma *et al.*, 1998). With continued maturation and position-dependent differentiation (Sürmeli *et al.*, 2011), expression of each transcription factor becomes progressively restricted to distinct SMN subtype identities, including medial, lateral, and hypaxial motor column subtypes. However, *in vitro* subtype-specific molecular refinements by heterogeneous ES-derived SMN are not distinct at early, immature stages of differentiation (Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; reviewed in Peljto and Wichterle, 2011).

More broadly, incomplete neuronal differentiation has been documented in protocols of largely spontaneous ES cell differentiation into retinal tissue and spinal cord nociceptors (Eiraku *et al.*, 2011; Nakano *et al.*, 2012; Chambers *et al.*, 2012). The remarkable differentiation of ES cells into self-organizing optic cup and retinal tissue recapitulates most aspects of retinal differentiation; however, photoreceptors within the spontaneously differentiated retina do not form the external rod segment and are not functional (Eiraku *et al.*, 2011; Nakano *et al.*, 2012). Similarly, human ES-derived nociceptors recapitulate multiple *in vivo* molecular and physiological characteristics, but the vast majority of these neurons *in vitro* are not responsive to capsaicin (Chambers *et al.*, 2012), a hallmark of mature nociceptors. Maturation deficits are also observed after directed differentiation of non-neuronal cell types; for example, established protocols for the directed differentiation of pancreatic islet beta cells generate endocrine cells that co-express glucagon and insulin, resembling immature precursors *in vivo* (Rezania *et al.*, 2012).

Remarkably, distinct features of neuronal maturation that are temporally correlated *in vivo* appear to be dissociable and independently regulated *in vitro*. For example, ES-derived SMN that are immature with regard to their expression of multiple subtype markers are, paradoxically, sufficiently mature to target their axons to the ventral spinal nerve root and form synapses at neuromuscular junctions (Wichterle *et al.*, 2002; Peljto and Wichterle, 2011). Similarly, in ES-derived nociceptors, relatively mature physiology in the absence of capsaicin response reflects potentially uncoordinated maturation that is not normally observed *in vivo* (Chambers *et al.*, 2012). In light of these data, the simultaneous expression of CoupTF1 and Ctip1, and the absence of Bhlhb5 in ES-derived neocortical-like neurons (**Figure 2.6**) could alternatively be interpreted as a relatively mature area-specific phenotype, entirely dissociable from early-stage maturation deficits indicated by other metrics.

Together, these examples suggest that distinct aspects of neuronal maturation might be independently regulated, posing additional challenges for the refinement of these protocols and the interpretation of results. Moreover, these examples reflect the increasingly substantiated view that *in vitro* environments pose multiple challenges for ensuring the appropriate maturation of ES-derived neurons. Though these deficits are a potentially major barrier to the directed differentiation of distinct neuronal subtypes, their identification might guide future refinements of these protocols.

5.3. Maturation-stalled neurons are potentially "confused"

Judging from the typically exceptional specificity of neuronal subtype involvement in specific neurodegenerative diseases, particularly ALS (*e.g.*, CSMN/SCPN in the brain, along with SMN in the spinal cord), the utility of directed differentiation for studying pathologic mechanisms and potential therapies hinges on its close approximation to *in vivo* development. Similarly compelling arguments maintain that directed differentiation to near-biological equivalents of neocortical neurons is critical to sufficiently model the basic biology of corticogenesis *in vitro* (Gaspard and Vanderhaeghen, 2010; Tiberi *et al.*, 2012).

Therefore, a major unanswered question, broadly relevant in the field, is whether or not there exist *in vivo* equivalents to some of these heterogeneous, maturation-stalled, ES-derived neocortical-like neurons. If not, the possibility remains that these ES-derived neurons, rather than "stalling" an appropriate developmental trajectory of maturation, are somehow "confused" with regard to molecular and functional identity and therefore might not be useful for neocortical neuronal subtype-specific disease modeling.

Increasingly, characterizations of ES cell directed differentiation protocols, exemplified by my findings and related findings by other groups, are consistent with the interpretation that neuronal identities can be potentially "confused" during spontaneous *in vitro* ES cell differentiation in culture. To help understand the nature of such developmental defects, it is useful to consider a popular model for differentiation in the field, called Waddington's 'epigenetic landscape' model, based on the work of C.H. Waddington (1957) (reviewed in Hochedlinger and Plath, 2009). In the Waddington model, a ball rolling down a hilly landscape represents the trajectory and progressive restriction of cell fate determination; the landscape encompasses a complex mix of stochastic and directed mechanisms of sequential cell fate

determination. Theoretically, this landscape could be altered, as might occur during the absence or mis-expression of specific transcriptional regulators, ultimately leading neurons down alternative developmental trajectories that would never occur *in vivo* and therefore appear "confused."

Questions about the fidelity of neocortical neuronal identity acquisition are increasingly relevant to the recent field of "direct reprogramming" to induced neuronal (iN) cells (reviewed in Vierbuchen and Wernig, 2011; Yang *et al.*, 2011). These iN cells can be generated by reprogramming non-neural mitotic cells, including heterogeneous embryonic tissue (Vierbuchen *et al.*, 2010), albumin-expressing liver cells (Marro *et al.*, 2011), or human fibroblasts (Pang *et al.*, 2011), using constitutive high expression of normally transiently expressed pro-neural transcription factors (see also: Son *et al.*, 2011; Caiazzo *et al.*, 2011). Not surprisingly, terminal differentiation is often incomplete, owing to residual epigenetic marks from the original cell type (Marro *et al.*, 2011; Vierbuchen and Wernig, 2011; Caiazzo *et al.*, 2011; Sadegh, unpublished data, 2012). Therefore, iN cells are likely "confused," seemingly lacking *in vivo* equivalents.

The possibility of *in vitro* ES-derived neuronal identity "confusion" is further supported *in vivo* by the identification of mixed neocortical neuronal molecular identities *in vivo* following the disruption of critical transcriptional regulators. For example, Sox5 is a post-mitotic determinant of the timing of CFuPN subtype acquisition; in its absence, normally early-born subplate neurons and CThPN shift their axonal projection patterns to resemble later-born SCPN, while maintaining "hybrid" expression of layer-specific markers (Lai *et al.*, 2008; Kwan *et al.*, 2008). Similarly, in the absence of Ctip1, Foxg1 or CoupTF1, the timing of neuronal entry into the cortical plate can be stalled, with late-arriving neurons adopting mixed laminar and subtype identities (Miyoshi and Fishell, 2012; Alfano *et al.*, 2011; Woodworth *et al.*, unpublished data, 2013). Finally, as discussed in Chapter 3, the overexpression of Fezf2 in

alternate neuron populations *in vivo* does not completely specify SCPN fate; these "hybrid" SCPN-like neurons do not activate the expression of Ctip2, nor fully repress the expression of Satb2 (Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2013; De la Rossa *et al.*, 2013). While the relevance of "hybrid" neocortical neuronal differentiation in disease modeling is unclear, it seems reasonable to question the present utility of potentially "confused" neurons and instead focus on developing refined protocols of differentiation.

Alternately, if ES-derived neocortical-like neurons closely resemble equivalent type(s) of neurons *in vivo*, one might speculate that *in vitro* differentiation conditions somehow bias neuronal differentiation toward theoretically transient or unknown subpopulations of neocortical neurons. This argument has been previously invoked to explain discrepancies between the differentiation of human retinal development and ectopic photoreceptor progenitors in human ES-derived retinal tissue (Nakano *et al.*, 2012). Importantly, there are precedents in neocortical biology for transient neuronal populations expressing characteristic markers of multiple neocortical subtypes, including subplate neurons (Hoerder-Suabedissen *et al.*, 2009; Ayoub and Kostovic, 2009) and Cajal-Retzius cells. Cajal-Retzius cells, in particular, are marked by the expression of Reelin and Tbr1, are generated in the periphery of the pallium, migrate within the neocortex, and occupy layer I until their apoptosis by the end of the first postnatal week (Soriano and Del Rio, 2005). Ultimately, it is difficult to argue in support of the hypothesis that seemingly "confused" ES-derived neocortical-like neurons represent a rare or transient *in vivo* equivalent neuronal population, in the absence of a comprehensive molecular taxonomy of neocortical neuronal subtypes. Moreover, the usefulness of these hypothetically transient or rare neuronal populations for subtype-specific biological and disease modeling would still be in question.

5.4. Potentially aberrant mechanisms of cell-intrinsic and cell-extrinsic CFuPN differentiation

Chromatin remodeling in ES-derived neocortical subtype refinement

During the sequential and progressive generation of neocortical projection neuron classes, types, and subtypes, the refinement and maturation of neocortical progenitors and post-mitotic neurons are progressively shaped by multiple epigenetic changes, including histone remodeling, histone methylation, DNA methylation, and regulation by non-coding RNA (reviewed in MacDonald and Roskams, 2009; Shimomura and Hashino, 2013). The roles of histone deacetylases have been identified in multiple forebrain cell types, including retinal subtypes (Chen and Cepko, 2007), and in neocortical neurons (Kishi and Macklis, 2004; MacDonald and Roskams, 2008). As discussed in Chapter 3, a number of neocortical subtype-specific transcriptional regulators (*e.g.*, Ctip2, Satb2, Ski) can directly interact with chromatin remodeling enzymes, implicating epigenetic mechanisms for neocortical subtype specification.

Taking advantage of the concept of "epigenetic priming" (reviewed in Papp and Plath, 2013) as an approach to generate a more permissive molecular context prior to Fezf2-directed differentiation, I screened a small library of compounds that modulate chromatin remodeling enzymes. I identify Sirt1 as the first chromatin-remodeling enzyme that is differentially expressed by two neocortical projection neuron subtypes *in vivo*: low in subcerebral projection neurons (SCPN), and high in inter-hemispheric callosal projection neurons (CPN) (**Chapter 3**). Moreover, Sirt1 is functional in the differential molecular refinement of neocortical SCPN and CPN subtype identity (**Figure 3.8, Figure 3.9**) and can enhance Fezf2-mediated SCPN directed differentiation of ES cells (**Figure 3.5**). My findings suggest that directed chromatin remodeling

potentially bypasses some of the deficits of ES-derived neocortical-like differentiation identified in Chapter 2 and discussed in Section 5.3.

Aberrant chromatin modifications might hinder CFuPN specification

A growing understanding of chromatin remodeling mechanisms enables a potential mechanistic understanding for the sequential and "nested" differentiation of forebrain, neocortical, and CFuPN progenitors (Woodworth *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013; **Chapter 4**). In ES cell differentiation, promoters of transcriptional regulators important for neocortical development are marked with "bivalent domains," which consist of hybrid (mix of active and repressive) histone modifications that are "poised" for activation; these bivalent domains ultimately resolve their histone modifications to an either completely active or completely repressed state (Bernstein *et al.*, 2006; Azuara *et al.*, 2006). The nested differentiation of CFuPN might be explained by the important role of "pioneer" transcription factors, which can occupy promoters harboring bivalent domains and maintain the poised state until these loci are fully activated during later stages of differentiation (*e.g.*, in hematopoietic differentiation, Oguro *et al.*, 2010; reviewed in Zaret and Carroll, 2011). These mechanisms of poised histone modifications might enable the maturation of neocortical subtypes.

To address whether bivalent domains are involved in the differentiation of mouse ES cells to neural fates, Bernstein, Lander, and colleagues at the Broad Institute confirmed the resolution of such bivalent domains in the neural differentiation of mouse ES cells (Mikkelsen *et al.*, 2007), using an embryoid body protocol that spontaneously generates Nestin/Pax6 co-expressing, non-telencephalic progenitors under the maintenance of EGF/FGF (Conti *et al.*, 2005). When ES cells are differentiated to neural precursor cells, multiple highly methylated

promoter regions (*e.g.*, including Sox2, Lhx2, Pax6, Sox6, CoupTF1) transition from having bivalent, "poised," domains to fully active sites; other promoter regions (*e.g.*, Ctip2, Satb2) are partially-active sites; lastly, some promoter regions (*e.g.* Fezf2, Ctip1, Bhlhb5, Otx1, Emx1) transition from bivalent domain to repressed sites. These changes are accompanied by proportional changes in the dose of gene expression (Mikkelsen *et al.,* 2007, Supplementary Figure 5). Although these findings are consistent with my findings of incomplete neocortical specification by ES cells (Chapter 2, Chapter 3), because these data were acquired in a non-telencephalic protocol of differentiation, the interpretations cannot be fully extended to neocortical development. However, recent evidence suggests that similar bivalent domains exist at the stage of E11.5 neocortical progenitors, specifically in the regulation of Ngn1 expression (Onoguchi *et al.*, 2012), raising the possibility that these mechanisms are indeed acting in neocortical development and potentially responsible for deficits in protocols of directed differentiation.

I speculate that during the directed differentiation of ES cells into maturation-stalled neocortical-like neurons, many of these "poised" histone modifications might not resolve to active sites. Importantly, deficits in these mechanisms are useful to understand why ES-derived cells, following spontaneous differentiation, might not have a sufficiently permissive molecular context for directed differentiation. In this dissertation, I have demonstrated that broad, non-specific manipulation of chromatin remodeling in ES cell differentiation (*e.g.*, valproic acid; Juliandi *et al.*, 2012) might potentially be replaced by more focal chromatin remodeling strategies (*e.g.* SCPN-specific Sirt1 inhibition; **Chapter 3**). In the future, further refinements to this approach might identify critical "pioneer" transcription factors that can recruit poised transcriptional domains and direct differentiation of neocortical neuronal subtypes (*e.g.*, by expression of CFuPN transcriptional regulators; **Chapter 4**).

Absence of critical factors and post-translational modifications might hinder CFuPN differentiation

As a consequence of deficits in ES-derived neocortical-like neuronal molecular context and chromatin remodeling (**Chapter 3**), I speculate that Fezf2-specific cofactors are absent following monolayer ES cell telencephalic differentiation. The most striking evidence for Fezf2 context dependence is the requirement of Sox11 and Sox4 for the neocortex-specific expression of Fezf2 (Shim *et al.*, 2012). Borrowing from examples in neocortical development, multiple cofactors might be required to form complexes that enable specific transcriptional activity. For example, the absence of either component of the transcriptional repressor complex containing Bhlhb5 and Prdm8 remarkably results in similar corticospinal tract defects (Joshi *et al.*, 2008; Ross *et al.*, 2012). In the absence of a full complement of transcriptional cofactors, one might bypass this requirement by "engineering" the ability of a single transcription factor to directly activate or repress its transcriptional targets in the absence of cofactors. Such a strategy, involving the fusion of direct activator or repressor domains, has been previously used to dissect Ngn2 functions (Kovach *et al.*, 2012). Hypothetically, if Fezf2 induces SCPN identity by cofactor-dependent repression of Fezf2 downstream targets, the "direct" repression of its targets might enable cofactor-independent induction of SCPN. However, preliminary data for the *in utero* mis-expression of chimeric Fezf2 proteins, fused to direct activator or repressor domains, suggests that the role of Fezf2 in promoting SCPN identity is neither completely based on repression or activation and likely requires distinct cofactors (Sadegh, unpublished data, 2011).

In addition, post-translational modifications (*e.g.* phosphorylation, SUMOylation) of critical transcription factors might be important for the regulation of subtype specification, and possibly for directed CFuPN differentiation. For example, Ngn2 has multiple phosphorylation

sites, the regulation of which determines its role in morphology or neuronal specification (Hand *et al.*, 2005; Ma *et al.*, 2008). Moreover, the SUMOylation of Ctip2, shown in hippocampal neurons (Tirard *et al.*, 2012), and Satb2, in non-neocortical cells (Dobreva *et al.*, 2003), might be important for regulating precise subtype specification in the neocortex. Thus, these post-translational modifications of critical transcriptional regulators are potentially useful for the assessment of appropriate neocortical neuronal specification from ES cells.

Extrinsic factors important in neocortical subtype differentiation

Given the incomplete progenitor specification, neuronal maturation, and subtype-specific neocortical differentiation from spontaneous ES cell differentiation by an established monolayer protocol (Chapter 2), multiple deficiencies might account for faulty differentiation. Numerous cell-extrinsic factors are required for neocortical development *in vivo* (reviewed in Tiberi *et al.*, 2012). Among these factors, a number of them are subtype-specific. First, the well-studied endo-cannabinoid signaling pathway has been recently identified to be critical for the maintenance of Ctip2 expression in layer V; CB1 receptor null mice resemble the Ctip2 null mice, with loss of expression and defasciculation of CSMN, whereas mice lacking a major cannabinoid-degrading enzyme display increased expression of Ctip2 in deep neocortical layers (Diaz-Alonso *et al.*, 2012). Second, IGF-1 is specifically required for extension, but not branching, of CSMN axons (Ozdinler and Macklis, 2006); moreover, multiple other factors, including IGF-2 and BDNF, enhance the survival of cultured CSMN (Dugas *et al.*, 2008). Finally, mutant mice lacking choroid plexus have a reduction in Ctip2-expressing layer V neurons (Johansson *et al.*, 2013). This phenotype is striking because it suggests that one or more of multiple growth factors secreted in the CSF (*e.g.* IGF-1, IGF-2; Lehtinen *et al.*, 2011) are critically important for the molecular refinement of CFuPN identity.

Multiple other extrinsic factors that are not subtype-specific might still be involved in the molecular refinement of CFuPN identity, particularly toward rostral motor cortex identity. First, mature cortical area pattern is organized during development by a finely tuned Fgf8 signaling gradient; the focal overexpression of Fgf8 by E11.5 neocortical progenitors can pattern rostral neocortical areas (Fukuchi-Shimogori and Grove, 2001; Assimacopoulos *et al.*, 2012). Importantly, Fgf8 was successfully applied to induce characteristics of rostral neocortical areas in an established embryoid body protocol of telencephalic differentiation (Eiraku *et al.*, 2008). Second, retinoids have been shown to promote neurogenesis in the mouse cerebral cortex (Siegenthaler *et al.*, 2009). Significantly, retinoids have been successfully applied to multiple protocols of directed differentiation to glutamatergic neurons from mouse ES cells (Bibel *et al.*, 2004) and to neocortical-like neurons from human pluripotent stem cells (Shi *et al.*, 2012), despite an early role of retinoids in caudal patterning of the neural tube. Differences in the timing and dosage of retinoid expression might be critical for establishing an appropriate molecular context of neocortical differentiation.

Though an established monolayer protocol of mouse ES cell differentiation (Gaspard *et al.*, 2009) offers advantages for detailed *in vitro* characterizations and potential mechanistic and therapeutic screening, the absence of three-dimensional cell-cell interactions in monolayer culture might impede subtype-specific refinements. Consistent with this hypothesis, an established embryoid body protocol of telencephalic differentiation generates neurons with seemingly reduced co-expression of distinct neocortical subtype markers (Eiraku *et al.*, 2008; Nasu *et al.*, 2012). Despite the apparent benefits of embryoid body culture, distinct deficits in ES-derived neuronal laminar organization indicate that cell-cell contacts are either incompletely replicated or insufficient in embryoid body culture without additional factors.

Notch signaling is one of the best-studied mechanisms of cell-cell signaling in the neocortex and is particularly relevant to directed differentiation given its longstanding roles in delineating distinct identities in related cell types (Greenwald and Rubin, 1992), including spinal cord motor neurons in zebrafish (Appel *et al.*, 2001). Notch signaling has emerged to be important for neocortical migration (Hashimoto-Torii *et al.*, 2008; Rodriguez *et al.*, 2012), neocortical neurogenesis (Mizutani and Saito, 2005), and asymmetric cell division in the neocortex (Rasin *et al.*, 2007; Bultje *et al.*, 2009). Notch expression oscillates in the neocortex, correlated with the timing of interkinetic nuclear migration (Kageyama *et al.*, 2008). Given the many roles of Notch signaling, it is important to note the molecular context of its regulation and the potentially varied distributions of its pathway components in different cell populations. This is more critical given that Notch signaling has at least two mechanisms, including lateral inhibition (Chitnis, 1995; Bray, 1998) and lateral induction (Ross and Kadesch, 2004).

Overall, multiple cell-intrinsic and cell-extrinsic processes, including focal chromatin remodeling, post-translational modifications of transcriptional regulators, paracrine molecular signaling, and cell-cell contacts, regulate the stepwise generation of CFuPN and diverse corticofugal types and subtypes *in vivo*. Importantly, deficits in some of these more specific mechanisms of differentiation might limit the *in vitro* capacity of ES cells to generate CFuPN. Therefore, identification of mechanistic deficits in differentiation protocols might indicate further directions for the generation of CFuPN and corticofugal subtypes.

5.5 Future directions: directed differentiation of clinically important CFuPN subtypes

As the primary population of output neurons in the neocortex, CFuPN enable direct communication to other regions of the central nervous system, including clinically important spinal cord networks of motor control. One corticofugal subtype, corticospinal motor neurons (CSMN), project long-distance axons to the spinal cord to control motor function (Stanfield, 1992). CSMN, and related sub-cerebral projection neurons (SCPN), selectively degenerate in the brains of both patients and mouse models (Zang and Cheema, 2002; Ozdinler *et al.*, 2011) of amyotrophic lateral sclerosis (ALS). After spinal cord injury (SCI), CSMN axonal damage is central to ensuing loss of motor function. Importantly, production of significantly large numbers of CFuPN / SCPN / CSMN will contribute to *in vitro* models of the strikingly specific neurodegeneration of CSMN during the extended pathogenesis of ALS.

To model human CFuPN degeneration in ALS *in vitro*, approaches of mouse ES cell directed differentiation would need to be adapted to other sources of pluripotent stem cells, including patient-derived induced pluripotent stem (iPS) cells. Since the first derivation of iPS from mouse (Takahashi and Yamanaka, 2006), and human fibroblasts (Takahashi *et al.*, 2007; Yu *et al.*, 2007), numerous protocol refinements and comparative epigenetics studies have indicated that ES and iPS cells are roughly equivalent pluripotent populations (Yamanaka, 2012). Moreover, an established monolayer protocol of pallial differentiation from mouse ES cells (Gaspard *et al.*, 2009) has been successfully adapted to human ES and iPS cells (Espuny-Camacho *et al.*, 2013; Shi *et al.*, 2012).

Using human iPS cells, large quantities of ALS patient-derived CSMN / CFuPN could be used for: 1) basic studies of mechanisms of neurodegeneration; 2) pharmacologic screening of small molecules and other bioactive compounds that might slow or reduce the neocortical component of ALS neurodegeneration; and 3) cellular replacement strategies using autologous

patient iPS-derived CSMN. First, recent *in vitro* studies have dissected mechanisms of ALS-specific neurodegeneration in spinal motor neurons (SMN), which are the "lower" motor neurons that selectively degenerate in the spinal cords of ALS patients and mouse models. These *in vitro* studies successfully recapitulate some, but not all, aspects of ALS pathogenesis by primary SMN (Nagai *et al.*, 2007) and by ES-derived SMN (Di Giorgio *et al.*, 2007; Di Giorgio *et al.*, 2008; Dimos *et al.*, 2008). Second, using these foundation data, hypothesis-driven approaches to drug screening are possible. Pharmacologic screening strategies are already being successfully applied to the development of potential treatments targeted toward disease-specific SMN. Work by the Inoue and Yamanaka labs identified a histone acetyl-transferase inhibitor that, in the context of increased metabolic stress, blocks degeneration of SMN derived from ALS patient-specific human iPS cells (Egawa *et al.*, 2012). In a complementary approach, Lee Rubin and colleagues conducted a high-content chemical screen and identified a kinase inhibitor that promotes the survival of SMN derived from ALS patient-specific human iPS cells (Yang *et al.*, 2013; see also Makhortova *et al.*, 2011). In a similar manner, large quantities of human iPS-derived CFuPN / SCPN / CSMN might also be used for high-content screening to identify small molecules or bioactive compounds that can reduce or slow the progression of CSMN-specific dysfunction in ALS. Third, the seemingly remote idea that ES cell-derived CSMN might be used for cellular replacement is supported by recent evidence that the generation, insertion, and functional integration of new neurons is possible in both neurogenic and non-neurogenic regions under specific conditions of the adult mammalian brain (Emsley *et al.*, 2004; Sohur *et al.*, 2006; Czupryn *et al.*, 2011; Wuttke and Macklis, unpublished data, 2013). Because of the focal localization of CSMN in primary motor cortex and positioning on the convexity of the cerebral cortex, transplantation of a single population of human ES- or iPS-derived CSMN might be especially feasible.

Several hypothetical approaches for *in vitro* directed differentiation of human ES- or iPS-derived CSMN are based on more closely mimicking an emerging molecular "logic" of *in vivo* CSMN development (Custo Greig *et al.*, in editorial revision, 2013). Because CSMN development follows a sequential and highly context-dependent process, hypothetical approaches include further refinements of progenitor-stage cell-extrinsic signaling and three-dimensional cell-cell contacts, and are complemented by the recapitulation of focal epigenetic processes and "nested" transcriptional regulation. By more closely applying *in vivo* developmental mechanisms, these *in vitro* approaches might avoid the generation of hybrid neuronal identity and maximally ensure the near biological-equivalency of ES- and iPS-derived CSMN based on molecular markers. Further characterization of ES-derived neuron physiology (*e.g.* channelrhodopsin and glutamate uncaging experiments in slice culture) would be essential to determine functional CSMN subtype maturation and specificity.

Importantly, because these hypothetical approaches would additionally generate numerous other neuronal subtypes, particularly given the asynchronous and heterochronic progression of ES cell differentiation, complimentary approaches for the prospective identification and isolation of CSMN would be an essential component of future protocols. Without a known context-independent CSMN-specific marker, prospective isolation of CSMN might be based on intersectional fate mapping technologies (Dymecki *et al.*, 2010) using multiple CSMN-specific markers (Arlotta *et al.*, 2005). Prior to the prospective isolation of CSMN, ES cells lines that report the expression of Fezf2 or Cux2 (Franco *et al.*, 2012; Custo Greig *et al.*, in editorial revision, 2013) might be employed to positively select precursors of CFuPN or CPN, respectively; such efforts are currently underway by other laboratories (Ruby and Zheng, 2009; Kmet *et al*., 2013). These hypothetical strategies are supported by my approaches of CFuPN-directed differentiation in this dissertation and from work more broadly in the field.

In this dissertation, I introduced distinct approaches to direct CFuPN differentiation from mouse ES cells, applying an emerging "logic" of stage-specific transcriptional regulation that is "nested" within neocortical classes, types, and subtypes. Although CSMN-specific differentiation remains elusive, findings presented in this dissertation indicate the feasibility of promoting CFuPN class, SCPN type, and potential CSMN subtype differentiation. Moreover, ongoing work and future insights regarding CFuPN development (Woodworth *et al.*, unpublished data, 2013; Custo Greig *et al.*, unpublished data, 2013; Sahni and Macklis, unpublished data, 2013; Galazo and Macklis, unpublished data, 2013) will help refine these protocols of directed differentiation. Overall, the biological and technical approaches presented in this dissertation both rigorously characterize ES-derived neuronal identity and identify novel strategies to promote CFuPN differentiation. This work will enable further advances in the broader field of *in vitro* neurodegenerative disease modeling, and, in particular, toward potentially developing new treatments for ALS and SCI.

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